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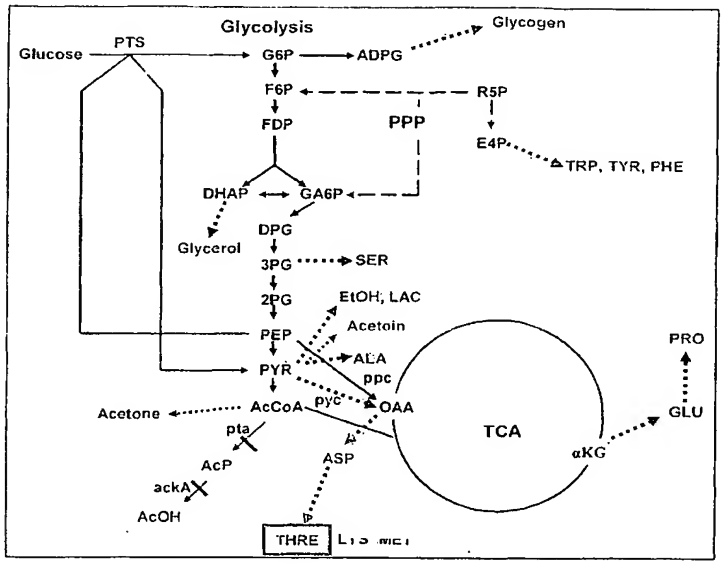
(54) Title: GENETICALLY ALTERED MICROORGANISMS WITH MODIFIED METABOLISM



(57) Abstract: The present invention relates to novel strains of microorganisms and fermentation processes involving these microorganisms. More specifically, the present invention relates to modified strains that direct overflow metabolism to the production of desired products and fermentation processed involving these microorganisms.

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GENETICALLY ALTERED MICROORGANISMS WITH MODIFIED METABOLISM

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] The present invention relates to novel strains of microorganisms and fermentation processes involving these microorganisms. More specifically, the present invention relates to genetically modified strains of microorganisms and the use thereof for the production of commercial products, such as recombinant proteins, nucleic acids, such as DNA, and amino acids. The present invention also relates to methods of preparing strains of microorganisms for such uses.

2. Description of Related Art

[0002] Microorganisms, such as bacteria, are used extensively in industrial processes to manufacture biopharmaceuticals, vaccine components, plasmid DNAs, vaccine DNAs and many specialty chemicals, including amino acids such as threonine. Bacteria used in industrial processes are typically grown in liquid medium supplemented with glucose as a source of carbon. Large amounts of glucose are required in industrial processes to grow bacteria to the high densities desired for maximizing volumetric productivity, for producing specialty chemicals containing carbon, and for maintenance of the bacteria. Bacteria uptake and assimilate glucose metabolites at a high rate. The flux of metabolites can be so high that it overwhelms one or more of the biochemical reactions in the central carbon pathways of the bacteria as the concentration of certain metabolites rise. The bacteria dispose of the high concentrations of metabolites by utilizing one or more "overflow" pathways. In *E. coli*, the predominant overflow pathway results in the production of acetate, which accumulates in the medium to toxic levels.

[0003] Although some types of bacteria specialized in mixed acid fermentations, such as *Lactobacilli*, are naturally resistant to or tolerant of high concentrations of acetate, it has been difficult to produce an acetate-resistant or acetate-tolerant *E. coli* due to the lack of specific transport proteins for acetate. The accumulation of acetate can be reduced somewhat by vigorous oxygenation of the growth medium, which increases the amount of glucose that can be handled by *E. coli* without overflow metabolism. Alternative ways of limiting overflow

metabolism include growing bacteria at lower temperatures to reduce the demand on the oxygen supply, adding glucose to the medium at a controlled rate throughout fermentation to ensure that the glucose concentration never rises above the critical concentration, or limiting nutrients to control oxygen demand. Each of these practical solutions suffers from the same drawback that they slow the growth of the bacteria, typically well below 25% of their maximum growth rate.

[0004] There have also been attempts to provide a genetic solution to the problem of overflow metabolism. The production of acetate in *E. coli* can be blocked by inactivating either of the two enzymes at the end of the overflow pathway: phosphoacetyltransferase (Pta) or acetate kinase (Ack) or both. However, the *pta* mutants grow more slowly and do not survive as well as the wild type under adverse conditions (Chang et al.), which was attributed to the accumulation of acetyl coA. Excess acetyl CoA in the Pta mutant of *E. coli* was consumed by introducing heterologous genes for the synthesis of poly-β-hydroxybutyrate, which yielded a strain with a growth rate only half as fast as the wild type. An alternative genetic approach involved increasing the flux from PEP to oxaloacetate by overexpressing PEP carboxylase using a heterologous promoter, which yielded a modified strain with a growth rate similar to the parent strain. By also introducing a fadR mutation to deregulate the glyoxylate shunt pathway, acetate accumulation in the strain was reduced four-fold without significantly reducing growth rate, however, the fadR mutation reduced the efficiency of glucose utilization and may compromise the survival or growth of the strain under certain conditions. Another genetic strategy to minimize acetate production involved mutating the PTS system responsible for the uptake of glucose from the medium to shift the uptake of glucose occurs to alternative, slower mechanisms. Although mutating the PTS system results in a lowered glucose flux such that acetate does not accumulate via the overflow pathway, the mutation also results in a lower growth rate. The current methods for minimizing overflow pathways also results in undesirable decreases in microorganism growth rates. Therefore, a need continues to exist for microorganism strains with modified overflow pathways and for methods of producing commercial products using microorganisms in order to reduce wastage and improve efficiency.

SUMMARY OF THE INVENTION

[0005] The present invention is related to a strain of a microorganism that exhibits one or more of the following properties in a growth medium comprising glucose: (i) a reduction in the flux of

production of a toxic metabolite by at least 50% compared to a wild type microorganism; (ii) at least 10% of the flux of glucose into glycolysis is flux to one or more alternative metabolites; and (iii) a growth rate of least 50% of the wild type microorganism. The strain may be an acetate sensitive bacterium. The acetate-sensitive bacterium may be *E. coli*. The strain may also be a reduced genome microorganism.

[0006] The strains may be grown in medium comprising at least 0.4%. Acetate may be the toxic metabolite which has reduced production by the strains. Alternative metabolites may be glycerol, glycogen, trehalose, saccharides, acetaldehyde, ethanol, lactate, formate, citric acid cycle metabolites, and L-amino acids. The citric acid cycle alternative metabolites may be oxaloacetate, citrate, succinate and succinyl-CoA. The L-amino acid alternative metabolites are selected from the group consisting of L-alanine, L-valine, L-leucine, L-aspartate, L-asparagine, L-lysine, L-methionine, L-threonine, L isoleucine, L-glutamate, L-glutamine, L-proline, L-arginine, L-tryptophan, L-tyrosine, L-phenylalanine, L-serine, L-glycine, L-cysteine, and L-histidine.

[0007] The genome of the strain may comprise a mutation in at least one gene encoding an enzyme in the acetate pathway. The genome of the strain may comprise a mutation in at least one promoter of a gene encoding an enzyme in the acetate pathway. The mutation may be a point mutation, a deletion, an insertion, and a rearrangement. The enzyme in the acetate pathway may be coded by a gene including, but not limited to, Ack and Pta.

[0008] The strain may comprise a heterologous gene encoding an enzyme that produces oxaloacetate. The strain may comprise a heterologous promoter operatively linked to a gene encoding an enzyme that produces oxaloacetate. The enzyme that produces oxaloacetate includes, but is not limited to, phosphoenolpyruvate kinase, phosphoenolpyruvate carboxylase and pyruvate carboxylase.

[0009] The strain may comprise an operon for production of an end metabolite operably linked with at least one non-native promoter. The end metabolite may be an L-amino acid including, but not limited to, L-alanine, L-valine, L-leucine, L-aspartate, L-asparagine, L-lysine, L-methionine, L-threonine, L isoleucine, L-glutamate, L-glutamine, L-proline, L-arginine, L-tryptophan, L-tyrosine, L-phenylalanine, L-serine, L-glycine, L-cysteine, and L-histidine.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figure 1 shows the metabolic pathways for glycolysis with enzymes present in *E. coli* K-12 MG1655 shaded.

[0011] Figure 2 shows the pathways for pyruvate metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0012] Figure 3 shows the metabolic pathways for the citric cycle (TCA cycle) with enzymes present in *E coli* K-12 MG1655 shaded.

[0013] Figure 4 shows the metabolic pathways for the pentose phosphate pathway with enzymes present in *E coli* K-12 MG1655 shaded.

[0014] Figure 5 shows the metabolic pathways for the synthesis and degradation of ketone bodies with enzymes present in *E coli* K-12 MG1655 shaded.

[0015] Figure 6 shows the metabolic pathways for valine, leucine and isoleucine biosynthesis with enzymes present in *E coli* K-12 MG1655 shaded.

[0016] Figure 7 shows the metabolic pathways for valine, leucine and isoleucine degradation with enzymes present in *E coli* K-12 MG1655 shaded.

[0017] Figure 8 shows the metabolic pathways for glutamate metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0018] Figure 9 shows the metabolic pathways for alanine and aspartate metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0019] Figure 10 shows the metabolic pathways for glycine, serine and threonine metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0020] Figure 11 shows the metabolic pathways for methionine metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0021] Figure 12 shows the metabolic pathways for cysteine metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0022] Figure 13 shows the metabolic pathways for lysine biosynthesis with enzymes present in *E coli* K-12 MG1655 shaded.

[0023] Figure 14 shows the metabolic pathways for lysine degradation with enzymes present in *E coli* K-12 MG1655 shaded.

[0024] Figure 15 shows the metabolic pathways for arginine and proline metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0025] Figure 16 shows the metabolic pathways for tyrosine metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0026] Figure 17 shows the metabolic pathways for phenylalanine metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0027] Figure 18 shows the metabolic pathways for tryptophan metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0028] Figure 19 shows the metabolic pathways for phenylalanine, tyrosine and tryptophan biosynthesis with enzymes present in *E coli* K-12 MG1655 shaded.

[0029] Figure 20 shows the metabolic pathways for histidine metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0030] Figure 21 shows the pathways for the urea cycle and the metabolism of amino groups with enzymes present in *E coli* K-12 MG1655 shaded.

[0031] Figure 22 shows the metabolic pathways for glycoxylate and dicarboxylate metabolism with enzymes present in *E coli* K-12 MG1655 shaded.

[0032] Figure 23 shows the entire genome of *E. coli* K-12. Origin and termination of replication, genes for r-RNA (red), t-RNA (green) and small regulatory RNA genes (blue) and the known positions of IS and RHS sequences in the outer two rings. The next 4 rings show alignments of the *E. coli* pathogen genomes with K-12. Large k-islands are vividly apparent at this scale introgressed into the backbone. Deletion endpoints of MDS12 which remove the largest k-islands, additional deletions of MDS40 and planned additional deletions are shown in red, green and blue respectively. The next two circles (red) show genome hybridization of MDS40 genomic and RNA expression ratios done with *E. coli* Affymetrix GeneChips. The overall structure of MDS40 is confirmed both by the absence of genomic DNA hybridization and lack of gene expression from the areas covered by the deletions. RNA expression levels are from log phase cells growing in minimal medium (green MDS40 and MG1655). The inner rings ring show the orientations of the open reading frames of *E. coli* and the innermost ring indicates those genes for which sce-poson mutants are available.

[0033] Figure 23 shows the growth profiles of MDS41-R13, MG1655 and W3110.

[0034] Figure 24 shows the results the properties of reduced genome strains.

[0035] Figure 25 (upper panel) shows the OD (circles), acetate concentration (diamonds) and glucose concentration (triangle) as a function of time for MDS42. Figure 25 (lower panel) shows the OD (circles) and specific CAT activity as a function of time for MDS42.

[0036] Figure 26A shows the OD (circles) and ln(OD) as a function of time for MDS42-pta. Figure 26B shows glucose concentration as a function of time for MDS42-pta. Figure 26C shows specific CAT activity (squares) and acetic acid concentration (diamonds) as a function of time for MDS42-pta.

[0037] Figure 27 shows a comparison of threonine production of MDS42- and MG1655-based strains

[0038] Figure 28 demonstrates the expression of recombinant proteins from *A. thaliana* in threonine-producing strains.

[0039] Figure 29 shows pathways that may be regulated to control overflow metabolism in the production of a recombinant protein.

[0040] Figure 30 shows a comparison of growth of MG1655 to MG1655Δ*iclR*- during fermentation on glucose.

DETAILED DESCRIPTION OF THE INVENTION

[0041] The present invention provides modified strains of microorganisms with improved growth rates in a medium comprising of a carbon source, such as glucose. The strains are modified to reduce the production of toxic overflow metabolites, such as acetate. The production of toxic overflow metabolites is reduced by (i) blocking production of one or more toxic overflow metabolites, (ii) enhancing the conversion of upstream metabolites to alternative metabolites or a combination thereof. By enhancing the conversion of upstream metabolites, the strains may be able to prevent the accumulation of upstream metabolites resulting from the block in the production of the toxic overflow metabolite. The strains may be used to produce recombinant proteins, nucleic acids, such as DNA, specialty chemicals or other products of interest at increased rates of efficiency based on the modifications described herein.

1. Definitions

[0042] As used herein, the term "alternative metabolite" means a metabolite, other than the toxic overflow metabolite, that is derived from an upstream metabolite. Representative examples of

alternative metabolites include glycerol, acetaldehyde, ethanol; lactate; formate, citric acid cycle metabolites, and L-amino acids.

[0043] As used herein, the term "block," when used in reference to a metabolite, means that the production of the metabolite may be reduced in a strain by any method that leads to a reduction in the cellular concentration of said metabolite. The cellular concentration of the metabolite may be reduced by methods including, but not limited to, (i) an increase in the export of the metabolite from the intracellular space of the strain; (ii) introduction of a feedback inhibition system; and, (iii) a block in cellular activity of an enzyme in the synthetic pathway of the metabolite.

[0044] As used herein, the term "block," when used in reference to an enzyme or gene encoding an enzyme, means that the cellular activity of the enzyme is reduced. The cellular activity of an enzyme may be reduced by any methods including, but not limited to, reduced transcription, reduced translation or mutagenesis of a gene coding for said enzyme. Transcription may be reduced by mutagenesis of the gene encoding the enzyme and associated regulatory sequences. Translation may be reduced by any manner including, but not limited to, antisense, RNAi, and mutagenesis of the coding sequence and associated regulatory sequences.

[0045] As used herein, the term "end metabolite" means an alternative metabolite that may be stored or exported by the stain.

[0046] As used herein, the term "enzyme" means a protein which acts as a catalyst to induce a chemical change in other compounds, thereby producing one or more products from one or more substrates. Enzymes are referred to herein using standard nomenclature or by their EC number, as recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology as of March 11, 2004.

[0047] As used herein, the term "inactivate" or "inactivating," when used in reference to an gene or regulatory element, means that the gene is blocked or that the activity of the regulatory elements is reduced or eliminated.

[0048] As used herein, the term "introduce" or "introduced," when used in reference to adding a gene to a strain, means that the gene may be integrated into the chromosome of the strain or contained on a vector, such as a plasmid, in the strain.

[0049] As used herein, the term "maximum tolerated % concentration of glucose" refers to the concentration of glucose at which the growth rate or other property of a microorganism (e.g.,

production of a product of interest) is substantially reduced under certain conditions. The maximum tolerated % concentration of glucose is a function of many factors including, but not limited to, strain of microorganism, cell density, temperature and oxygen concentration. One of ordinary skill in the art is familiar with these factors and others, as well as with the maximum tolerated % concentration of glucose under various conditions. The maximum tolerated % concentration of glucose may be from 1% to 10% w/v glucose, including but not limited to, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8% or 9%.

[0050] As used herein, the term "mutagenesis" means any modification to the nucleic acid of a parent strain. Mutagenesis of nucleic acid may be of any type including, but not limited to, deletion, insertion, substitution, rearrangement, and suppressor and point mutations.

[0051] As used herein, the term "overexpressing" means that the total cellular activity of protein encoded by a gene is increased. The total cellular activity of a protein may be due to increased cellular amounts of the wild-type protein, or a modified protein with increased activity compared to the parent, or increased half-life of the protein. Total cellular amounts of a protein may be increased by methods including, but not limited to, amplification of the gene coding said protein or operatively linking a strong promoter to the gene coding said protein.

[0052] As used herein, the term "protein," means peptides, polypeptides and proteins, whether native or recombinant, as well as fragments, derivatives, homologs, variants and fusions thereof.

[0053] As used herein, the term "toxic overflow metabolite" means a metabolite that causes a reduction in the growth rate of a parental strain by more than 10%, 20%, 30%, 40% or 50% under conditions of a high concentration of carbon source and low concentration of oxygen. For each level of oxygenation, there is a critical concentration of glucose, above which overflow metabolism occurs and toxic overflow metabolites accumulate. A representative example of a toxic overflow metabolite is acetate. The relationship between the rate of carbon source uptake and oxygen concentration is well known to those of skill in the art, and is discussed in Akesson et al. 1999.

[0054] As used herein, the term "upstream metabolite" means a metabolite that is a precursor to toxic overflow metabolites and alternative metabolites.

2. Blocking Toxic Overflow Metabolites

[0055] The strains of the present invention are modified to comprise one or more blocks that reduce the production of one or more toxic overflow metabolites. The strain may be modified to block the production of acetate. The production of acetate may be blocked by blocking acetate kinase (Ack), phosphoacetyltransferase (Pta), or both using methods known to those of skill in the art.

[0056] A homolog of *eutI* in *Salmonella typhimurium* displays Pta-like activity. As a result the presence of *eutI*, or a homolog thereto, may contribute to acetate formation. In order to block the production of acetate, *eutI* or its homolog may be blocked.

[0057] Compared to the parent strain, the strains of the present invention have reduced flux of acetate production when grown in the presence of an abundance of a carbon source. When grown in liquid medium comprising from 0% to the maximum tolerated % concentration of glucose, the strains of the present invention may have a flux of acetate production reduced by amounts including, but not limited to, at least about 30% to about 100% as compared to the parent strain. At such conditions, a strain may have a flux of acetate production decreased by about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% as compared to a wild type microorganism.

3. Enhancing Conversion of Upstream Metabolites

[0058] The strains of the present invention are also modified to increase the rate of conversion of one or more upstream metabolites to alternative metabolites. Blocking production of a toxic metabolite by itself may cause an accumulation of upstream metabolites, which can be toxic in their own right at elevated concentrations. By blocking the overflow metabolite in combination with increasing the conversion of the upstream metabolites to alternative metabolites, the strains of the present invention may be able to grow at enhanced rates.

[0059] The rate of conversion of upstream metabolites into alternative metabolites may be increased by methods including, but not limited to, expressing one or more heterologous genes, overexpressing one or more endogenous genes, or a combination thereof, wherein the enzymes encoded by said genes individually or in sequence with other enzymes convert the upstream metabolites into alternative metabolites. The alternative metabolites include, but are not limited to, metabolites of glycolysis, the TCA cycle, or downstream metabolites thereof. Directing

carbon metabolism towards the TCA cycle may be used to replenish metabolites that are used to make useful chemicals or which are the sources of amino acids for recombinant proteins. Enzymes that may be used to convert upstream metabolites into alternative metabolites include, but are not limited to, those enzymes listed on Figures 1-21.

[0060] Preferred enzymes that may be used to convert upstream metabolites into alternative metabolites include, but are not limited to, phosphoenolpyruvate carboxylase (EC 4.1.1.31), phosphoenolpyruvate carboxykinase (EC 4.1.1.32 and EC 4.1.1.38), ribulose-bisphosphate carboxylase (EC 4.1.1.39), pyruvate kinase (EC 2.7.1.40), pyruvate, phosphate dikinase (EC 2.7.9.1), pyruvate, water dikinase (EC 2.7.9.2), pyruvate carboxylase (EC 6.4.1.1)

[0061] The rate of conversion of upstream metabolites into alternative metabolites may also be increased by deregulating the glycoxylate shunt pathway and modifying the expression of enzymes in the pentose phosphate pathway (Figure 4).

[0062] The strains may have increased rates for converting pyruvate, PEP and acetyl CoA to oxaloacetate, which may be used, for example, to avoid the build up of upstream metabolites from the blocked acetate pathway. Increased conversion of pyruvate, PEP and acetyl CoA to oxaloacetate may be produced by methods such as overexpressing PEP kinase, overexpressing PEP carboxylase, or overexpressing pyruvate carboxylase. PEP kinase and PEP carboxylase lead to the production of oxaloacetate from PEP, whereas pyruvate carboxylase converts pyruvate to oxaloacetate. A buildup of acetyl CoA that may arise from the acetate block may be eliminated by endogenous or increased levels of citrate synthetase which combines acetyl CoA and oxaloacetate to form citrate.

[0063] Pyruvate carboxylase may be introduced from a heterologous source if the microorganism, such as *E. coli,* does not naturally have this enzyme. Sources of heterologous pyruvate carboxylase include, but are not limited to, *Rhizobium entli* [4] and *Corynebacterium glutamicum* [US 2003 0027305]. The pyruvate carboxylase enzyme may be resistant to feedback inhibition by products further down the metabolic pathway [US 2002 0177202].

[0064] The strains of the present invention have flux to one or more alternative metabolites of at least from about 2% to about 60% of the flux of glucose into glycolysis. A strain may have a flux to one or more alternative metabolites of at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55% or 60% of the flux of glucose into glycolysis.

4. Production of Non-Toxic End Metabolites

[0065] The strains of the present invention may also be modified to increase the flux from upstream metabolites to end metabolites. The combination of blocking a toxic overflow pathway and redirecting overflow metabolism to non-toxic end metabolites, may take care of excess metabolites created by an imbalance of carbon source, such as glucose, and the maximum attainable oxygen uptake, thereby enabling the strain to grow at maximal rates. The different modifications may be regulated to control the redirection of overflow metabolites while ensuring optimal availability of intermediates for synthesis of optional products in addition to the non-toxic end product.

[0066] The types of modifications used to divert upstream metabolites to end metabolites includes, but is not limited to, (a) blocking feedback inhibition mechanisms that block synthesis of the end metabolite or precursors thereof when the normal cellular needs have been met; (b) increasing the amount of or activity of mechanisms that export the end metabolite or precursors thereof through the cell membrane into the medium; (c) blocking pathways that cause re-uptake of the end metabolite or precursors thereof through the cell membrane from the medium; and (d) blocking pathways that degrade the end metabolite or precursors thereof. There are several ways that each of these modifications may be achieved, which will be well understood by one of ordinary skill in the art. For example, resistance to feedback inhibition may be achieved by using a mutant resistant to an analog of the end metabolite and also screening for overproduction of the end metabolite. At the transcriptional level, relief of feedback repression may be achieved by changing the promoter and any upstream sequence coding for an attenuator for an alternative promoter that may be inducible or constitutive.

[0067] The end metabolite may be exported into the medium or retained intracellularly. The end metabolite may be any metabolite that is non-toxic, or that tolerance or resistance to its presence in higher than normal concentrations can be induced. The end metabolite may be any metabolite including, but not limited to, those metabolites listed in Figures 1-21. Representative examples of end metabolites include any metabolite that may be ultimately derived from pyruvate, PEP, acetyl CoA, metabolites of glycolysis, metabolites of the TCA cycle, or downstream products thereof. End metabolites of overflow metabolism also include those listed in Table 1.

Table 1 - End Metabolites

Precursor	End Metabolite
Phosphoenolpyruvate	Succinate
Pyruvate	L-alanine; L-valine; L-leucine; acetaldehyde/ethanol; lactate; formate
Oxaloacetate	L-aspartate; L-asparagine; L-lysine; L-methionine; L-threonine; L-isoleucine
α-Ketoglutarate	L-glutamate; L-glutamine; L-proline; L-arginine
Erythrose-4-phosphate, phosphoenolpyruvate	L-tryptophan; L-tyrosine; L-phenylalanine
α-D-glucose-6-phosphate, glyceraldehyde-3-phosphate	L-histidine
3-phosphoglycerate	L-serine; L-glycine; L-cysteine

a. Amino Acids

[0068] The end metabolite may be an amino acid. Many amino acids are ideal end products because they are readily re-utilized as carbon sources, without excessive acidification of the medium or production of acetate. Amino acids may be produced as end metabolites of overflow metabolism from precursors including, but not limited to, those listed in Figures 1-4 and 6-21. Amino acids may also be produced as end metabolites of overflow metabolism from precursors including, but not limited to, those listed in Table 1.

1) Threonine, Aspartate, Asparagine, Lysine, Methionine and Isoleucine

[0069] The strain may produce L-threonine as an end metabolite of overflow metabolism. Threonine may be made from oxaloacetate via aspartate by enzymes that are coordinately controlled on a single operon. The threonine operon contains three genes: threA, B and C. The synthesis of threonine is normally tightly controlled to produce only the amount of threonine required to support cellular activity. If threonine begins to accumulate, the cell uses several mechanisms to reduce its synthesis. As a result, bacteria such as *E. coli* do not naturally synthesize excess quantities of threonine or export threonine into the medium. In order to make strains synthesize excess threonine, and preferably export it as a non-toxic overflow metabolite into the medium, one or more genetic modifications may be required.

[0070] Several combinations of modifications allow the ability to vary the amount of threonine that is synthesized and exported into the medium. The categories of modification that may be performed to produce and export threonine include, but are not limited to, (a) blocking the

feedback mechanisms that shut off threonine synthesis when the normal cellular needs have been met, either at the enzyme level, or at the transcription level; (b) increasing the amount of or activity of mechanisms that export threonine through the cell membrane into the medium; (c) blocking pathways that cause re-uptake of threonine through the cell membrane from the medium; (d) blocking pathways that degrade threonine; and (e) adding genes coding for enzymes that aid in the production of threonine including, but not limited to, aspartate semialdehyde dehydrogenase to assist in the conversion of aspartate to threonine. Representative examples of such modifications are discussed extensively in U.S. Patent No. 5,939,307, which is incorporated herein by reference.

[0071] The feedback control mechanisms of threonine can be blocked in a strain by (i) introducing a modified threA gene, which codes an enzyme that is resistant to feedback inhibition by threonine, from strains including, but not limited to, ATCC21277, ATCC21530 and strains prepared by mutagenesis; (ii) overcoming the action of the attenuator that down-regulates transcription of the operon when threonine is in excess, for instance by replacing the upstream part of the operon by a synthetic promoter construct, as described in U.S. Patent No. 5,939,307, which is incorporated herein by reference; and (iii) placing the threonine operon under the control of an inducible foreign promoter including, but not limited to, the ara, lac, tac, lambda PL or PR promoters.

[0072] Resistance to high concentrations of exogenous threonine in a strain can be induced by (i) producing mutant strains that are selected for growth on minimal medium containing threonine; (ii) amplification of genes coding for systems that cause increased excretion or elimination of xenobiotics, such as antibiotics; and (iii) overexpression of endogenous genes including, but are not limited to, the rhtA and rhtC genes [6] . The rhtA modification may be the rhtA23 modification (A to G replacement at position -1 relative to the ATG start codon), which leads to enhanced expression of rhtA by ten-fold [7].

[0073] The reuptake of threonine may be reduced or prevented in a strain by any method including, but not limited to, mutating cell-membrane permeases including, but not limited to, the LIVI pathway, livJ, serine-threonine-H+ permease, and tdcC. Degradation of threonine may be reduced or prevented by any method including, but not limited to, inactivation of the threonine dehydrogenase gene to prevent conversion of threonine to alpha-amino-beta-ketobutyrate.

[0074] If it is desired to have the strain re-utilize the threonine when the carbon source has been exhausted, the uptake and degradation pathways may be put under the control of a promoter that is subject to catabolic repression in the presence of the carbon source. Such a promoter would become derepressed when the carbon source is exhausted, thereby causing transcription of the permease and dehydrogenase genes which would cause the uptake and degradation pathways to become operational. Representative examples of catabolic promoters include, but are not limited to, the more than 100 catabolic promoters in *E. coli*. If the re-uptake of the threonine is not desired in the strain, the transporter that is responsible for threonine re-uptake may be inactivated.

[0075] Using the modifications described above for producing threonine, overflow metabolism may also be directed in a strain to produce related amino acid metabolites including, but not limited to, L-aspartate, L-asparagine, L-lysine, L-methionine and L-isoleucine.

[0076] L-lysine may be produced in a strain by modifying the gene coding for aspartate kinase III (lysC) such that the strain is resistant to feedback inhibited by lysine and also by inactivating the attenuator sequence upstream of the gene that is responsible for feedback repression. Alternatively, the aspartate kinase III gene may be placed under the control of an independent inducible promoter.

[0077] Overflow metabolism may be diverted in a strain to produce L-methionine by modifications to the gene for aspartate kinase II (metL). If resistance to feedback inhibition is not required, the strain may be modified to place metL under the control of an independent promoter, or remove the attenuator sequence. Methionine overproducing strains may also be produced by selecting for strains that are resistant to L-methionine analogs and screening to find methionine overproducers [8]. Alternatively, the methionine repressor gene (metJ) may be modified to introduce a substitution of serine-54 to asparagine in the encoded protein [8].

[0078] L-isoleucine may be produced in a strain by methods including, but not limited to, making the threonine deaminase feedback inhibition system resistant to isoleucine and introducing a gene for acetohydroxyacid synthase II [9]. The strain may produce other metabolites, which, if undesirable for a particular industrial application, may be eliminated by introduction of additional genes including, but not limited to, dihydroxyacid dehydratase and transaminase B [9].

2) Alanine, Valine and Leucine

[0079] L-alanine, L-Valine and L-Leucine may be produced in a strain using pyruvate as a precursor. The strain may be modified to comprise L-alanine dehydrogenase, for example, from *Bacillus sphaericus* [10]. Using similar modifications, overflow metabolism may also be directed in a strain to produce related amino acid metabolites including, but not limited to, L-valine and L-leucine.

3) Proline, Glutamate, Glutamine and Arginine

[0080] Metabolism can also be diverted in a strain to produce L-proline, which may have the additional benefit of increasing the tolerance of the strain to osmotic stress. The gene (proB) coding for the enzyme (γ-glutamyl kinase) which is involved in the first step of proline biosynthesis may be modified to make it resistant to feedback inhibition by proline. A gene containing the mutation proB74 may also be introduced into the strain to replace the wild-type proB gene [11] or alternatively site-specific mutagenesis or homologous recombination of a synthetic gene fragment may be used to introduce suitable mutations that include substitution of an A for a G at position 319 in the coding strand, changing the amino acid at position 107 from an aspartate to asparagine. Another suitable mutation would be to cause a substitution of alanine-143 to glutamate. Other modifications that are suitable include selection of mutants that are resistant to 3,4-dehydro-DL-proline, a toxic analog of proline, and screening for a strain that overproduces proline [12].

[0081] Using similar modifications as described above for producing proline, overflow metabolism may also be directed in a strain to produce related amino acid metabolites including, but not limited to, L-glutamate, L-glutamine and L-arginine.

4) Tryptophan, Phenylalanine and Tyrosine

[0082] Overflow metabolism of the strain may also be diverted to produce L-tryptophan, which may be formed from PEP and erythrose-4-phosphate. The gene for the enzyme tryptophanase (tnaA) may be inactivated to prevent degradation of tryptophan, though this may not be desirable if it is desired to have the accumulated tryptophan re-utilized. Inactivation of this enzyme may also have the benefit of reducing the characteristic odor of the fermentation. In addition, the genes for feedback inhibition resistant enzymes that occupy key controlling steps in tryptophan synthesis may also be introduced into the strain including, but not limited to, anthranillate synthetase and phosphoribosyl anthranilate transferase as found in the plasmid pSC101.trp115

[13]. Additional mutations may increase tryptophan production in the strain, including inactivation of trpR [13]. Alternatively, strains with inactivated tryptophanase and strains resistant to tryptophan analogs, such as 4-, 5- or 6-fluoro-tryptophan or 4- or 6-methyl-tryptophan, may be used.

[0083] A similar strategy may be used to divert overflow metabolism to the production of L-phenylalanine. Feedback inhibition resistant chorismate mutase P-prephenate dehydratase (PheA) and 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (aroF) may be expressed, for example, from an inducible promoter system on a plasmid such as pSY130-14 [14].

[0084] Using similar modifications as described above for producing tryptophan and phenylalanine, overflow metabolism may also be directed in a strain to produce related amino acid metabolites including, but not limited to, L-tyrosine.

5) Cysteine, Serine and Glycine

[0085] Overflow metabolism may be diverted in a strain to produce L-cysteine. The strain may comprise modifications that inactivate the cysteine breakdown pathway. The strain may be produced by methods including, but not limited to, selecting a mutant that cannot grow on cysteine, or inactivating tnaA. A strain may also be made resistant to cysteine feedback inhibition by modifications including, but not limited to, (i) site directed mutagenesis of the gene coding for serine acetyltransferase (cysE) to substitute methionine-256 with isoleucine [15], alanine [16] or other amino acids; (ii) random mutagenesis and selection of overproducers [17]; and (iii) modifying cysE to delete the C-terminal end of the expressed protein [16]. The strain may also be modified to overexpress the yfiK gene [18], which codes for a cysteine transporter protein [19].

[0086] Using similar modifications as described above for producing cysteine, overflow metabolism may also be directed in a strain to produce related amino acid metabolites including, but not limited to, L-serine and glycine.

b. Non Amino Acid Metabolites

[0087] The end metabolite may also be substances in addition to amino acids. The end metabolite may be glycerol, which may be non-toxic in high concentrations and may be re-utilized. Furthermore, glycerol utilization may be naturally catabolite repressed in the presence of glucose. As soon as the glucose is exhausted, catabolite repression may be relieved, enabling the accumulated glycerol to be re-utilized. An advantage of producing glycerol as an end

metabolite is that further modifications to the strain may not necessarily be required to achieve well-controlled activation of re-uptake. Another advantage is that glycerol re-utilization may not generate acetate as an overflow metabolite.

[0088] A strain may be modified to accumulate glycerol by introducing heterologous genes coding glycerol phosphate dehydrogenase (GPD1) and glycerol-3-phosphate phosphatase (GPP2) under the control of a suitable promoter. Source of these genes include, but are not limited to, *Saccharomyces cerevisiae* (Progress Report, Contract QLK5-1999-01364, BioMat Project, European Commission, http://www.nf-2000.org/secure/FP5/F1185.htm). To achieve high levels of glycerol accumulation, endogenous triose phosphate isomerase may also be inactivated.

[0089] Strains may also be modified to divert overflow metabolism to a natural storage metabolite including, but not limited to, trehalose or glycogen. Glycogen may be synthesized by the polymerization of sugars and stored by the microorganism under certain circumstances, such as glycogenesis, and broken back down to constituent sugars when needed. The processes may be subject to coordinated control. A modification that may redirect overflow metabolism to cause glycogen accumulation may be to express the glycogen forming pathway, such as under control of an inducible promoter, such that it is uncoupled from normal mechanisms that control transcription. When the operon is turned off, the normal control may be reinstated, and the accumulated glycogen may be used as appropriate to the needs of the strain. For example, the gene (glgC) coding for the enzyme ADP-glucose synthetase (ADPG pyrophosphorylase) and the gene (glgA) coding for the enzyme glycogen synthase may be introduced into the cell under control of a suitable promoter. A suitable source would be plasmid pGT100. Another suitable modification may be to mutate glgQ to cause overproduction of the glycogen biosynthesitic enzymes. [20]. Optionally, the gene (glgP) for the glycogen branching enzyme may also be expressed under the control of a synthetic promoter. Additionally, a modification may be made to the glgC gene to substitute arginine-67 in the expressed protein to cysteine which may render the enzyme ADP-glucose synthetase less sensitive to its allosteric modulators and thereby less sensitive to control by normal metabolites. A source for the modified gene is also the *E. coli* mutant strain CL1136 [21] or the actual mutation. An alternative modification is to substitute lysine-296 and glycine-336 in the expressed protein to glutamate and aspartate, respectively [22],

or to use the gene from the *E. coli* K12 mutant 618. Another possible modification would be to express the glycogen degradative enzyme glycogen phosphorylase coded by gene glgP [23].

[0090] Using similar methodologies, strains may also be modified to divert overflow metabolism to trehalose, saccharides, acetaldehyde, ethanol, lactate and formate.

5. Product of Interest

[0091] The strains of the present invention may be used to produce a product of interest at high rates of efficiency in media with an abundance of a carbon source. The product of interest produced by the strains may be one or more of any products including, but not limited to, chemicals, amino acids, vitamins, cofactors, nucleic acids, such as DNA, growth factors, proteins and intermediates thereof. The product of interest may be a product that is naturally produced by the strains. The product of interest may also be a non-natural product that is produced as a result of heterologous genes being added to the strains.

[0092] The product of interest may be intracellularly located in the strain. The product of interest may also be secreted into the periplasm of the strain. The periplasm may be beneficial for protein production, because: (i) recombinant human protein may be produced with the correct amino terminus, whereas those produced in the cytoplasm may begin with an additional methionine not present in the natural protein; (ii) many proteins may fold correctly in the periplasmic space (iii) the correct disulfide bonds may be formed in the periplasm; (iv) the periplasmic space may contain much less and far fewer proteins than the cytoplasm, simplifying purification (v) there may be fewer proteases than in the cytoplasm, which may reduce protein digestion and loss; and (vi) expressed proteins may be readily released with other periplasmic proteins by specifically disrupting the outer membrane, substantially free of the more abundant cytoplasmic proteins.

6. Microorganisms

a. Parent Strain

[0093] The strains of the present invention may be derived from any parent microorganism that is capable of producing a desired end metabolite, either naturally or after being modified as described herein. Representative microorganisms useful in the practice of the present invention are available from the American Type Culture Collection. Other representative microorganisms

are described in S.Y. Lee, "*High Density Culture of Escherichia coli*," Tibtech 14:98-103 (1996). Representative examples of the microorganism include, but are not limited to, yeast and bacteria, such as *E. coli*.

[0094] The microorganism may also be a reduced genome microorganism, such as a reduced genome bacteria. Much of the genetic information contained within the genome of a microorganism may be deleted without detrimentally effecting production of the end metabolite. Moreover, microorganisms with a reduced genome may be advantageous over other strains in the production of many end metabolites. For example, a reduced genome may lead to the production of fewer native products or lower levels thereof which may lead to less complex purification of end metabolite. In addition, microorganisms with a reduced genome may be less metabolically demanding and thus may produce the end metabolite more efficiently. Reduced genome bacteria are discussed in copending U.S. Patent Application No. 10/057,582, which is incorporated herein by reference. A reduced genome bacteria with limited production of natural periplasmic proteins may be beneficial for expressing recombinant proteins in the periplasm. Examples of suitable reduced genome bacteria strains include, but are not limited to, MDS12, MDS13. MDS39, MDS40, MDS41-R13, MDS41E, and MDS42. The reduced genome microorganism may also be derived de novo from genes and operons from one or more microorganisms.

b. Modification

[0095] The strains of the present invention may be prepared by any of the methods and techniques known and available to those skilled in the art. Illustrative examples of suitable methods for constructing the inventive strains include (i) mutagenesis using suitable agents such as NTG [5, 24]; (ii) gene integration techniques, mediated by transforming linear DNA fragments and homologous recombination; (iii) transduction mediated by the bacteriophage P1; (iv) transformation with vectors comprising heterologous genes, modified genes, or endogenous genes operatively linked to non-native promoters; (v) production of resistant or tolerant strains; (vi) site-directed mutagenesis; and (vii) PCR-based mutagenesis. These methods are well known in the art and are described, for example, in J. H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1972); J. H. Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1992); M. Singer and P. Berg, Genes & Genomes, University Science Books, Mill Valley, Calif. (1991); J. Sambrook, E. F. Fritsch and T. Maniatis, Molecular Cloning: A Laboratory Manual,

2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); P. B. Kaufman et al., Handbook of Molecular and Cellular Methods in Biology and Medicine, CRC Press, Boca Raton, Fla. (1995); Methods in Plant Molecular Biology and Biotechnology, B. R. Glick and J. E. Thompson, eds., CRC Press, Boca Raton, Fla. (1993); and P. F. Smith-Keary, Molecular Genetics of Escherichia coli, The Guilford Press, New York, N.Y. (1989), which are incorporated herein by reference.

c. Growth Rates

[0096] The strains of the present invention have improved growth rates when grown in the presence of an abundance of a carbon source. When grown in liquid medium comprising from 0% to the maximum tolerated % concentration of glucose the strains of the present invention may have a growth rate including, but not limited to, greater than about 25% to about 400% as compared to the parent of the strain. The strains may be grown in medium comprising glucose at a w/v of at least about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.5, 2%, 2.5, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5%, 8%, 8.5%, 9%, 9.5% or 10%. At such conditions, a strain may have a growth rate greater than about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 105%, 110%, 115%, 120%, 125%, 130%, 135%, 140%, 145%, 150%, 155%, 160%, 165%, 170%, 175%, 180%, 185%, 190%, 195%, 200%, 205%, 210%, 215%, 220%, 225%, 230%, 235%, 240%, 245%, 250%, 255%, 260%, 265%, 270%, 275%, 280%, 285%, 290%, 295%, 300%, 305%, 310%, 315%, 320%, 325%, 330%, 335%, 340%, 345%, 350%, 355%, 360%, 365%, 370%, 375%, 380%, 385%, 390%, 395%, or 400% as compared to a wild type microorganism.

d. Metabolic Flux

[0097] The strains of the present invention have improved metabolic flux when grown in the presence of an abundance of a carbon source. When grown in liquid medium comprising from 0% to the maximum tolerated % concentration of glucose, the strains of the present invention may have a metabolic flux including, but not limited to, from about 5% to about 90% of the carbon source being directed to the desired products. A strain may have a metabolic flux of about 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90%.

e. Production

[0098] The strains of the present invention may be able to produce end metabolites or other products of interest at higher rates. When grown in liquid medium comprising comprising from 0% to the maximum tolerated % concentration of glucose, the strains of the present invention may produce end metabolites or other other products of interest from about 0.001 g/L to about 100 g/L of the carbon source being directed to the desired products. A strain may have a metabolic flux of about 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80%.

f. Rate of Production

[0099] In view of the ability to control the amount of end metabolite produced as a result of overflow metabolism, as described above, the strains of the present invention may produce a variety of amounts of the end metabolite, at a variety of rates, and at variable rates of efficiency of carbon source utilization. The strains of the present invention may produce the end metabolite at least to levels including, but not limited to, about 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, 60 g/L, 70 g/L, 80 g/L, 90 g/L, and 100 g/L.

[0100] The strains of the present invention may produce the end metabolite at a rate including, but not limited to, at least about 0.50 g/L/hr, 0.75 g/L/hr, 1.00 g/L/hr, 1.25 g/L/hr, 1.50 g/L/hr, 1.75 g/L/hr, 2.00 g/L/hr, 2.25 g/L/hr, 2.50 g/L/hr, 2.75 g/L/hr, 3.00 g/L/hr, 3.25 g/L/hr, 3.50 g/L/hr, 3.75 g/L/hr, 4.00 g/L/hr, 4.25 g/L/hr, 4.50 g/L/hr, 4.75 g/L/hr, and 5.00 g/L/hr.

[0101] The strains of the present invention may produce the end metabolite at a rate of efficiency of carbon source utilitization including, but not limited to, at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, and 75%.

7. Fermentation

[0102] The strains may be used to produce desired products in batch fermentations, where the entire required amount of the carbon source may be added at the beginning of the fermentation. The strains may also be used to produce desired products in fed batch fermentations. The feeding rate of the carbon source may be any amount up to that which produces the maximum tolerated concentration of glucose but is preferably the minimum amount required to maintain

maximal growth rate. The strains may also be used to produce desired products in continuous or "chemostat" modes of fermentation, which may allow maintenance of a higher dilution rate.

[0103] The strains of the present invention may be used for fermentation processes in a synthetic or natural medium containing at least one carbon source and at least one nitrogen source that may be utilized by the strain by virtue of its possessing the necessary metabolic pathway(s) and, as appropriate, inorganic salts, growth factors and the like.

[0104] Illustrative examples of suitable carbon sources include, but are not limited to: carbohydrates, such as glucose, fructose, sucrose, starch hydrolysate, cellulose hydrolysate and molasses; organic acids, such as acetic acid, propionic acid, formic acid, malic acid, citric acid, and fumaric acid; and alcohols, such as glycerol.

[0105] Illustrative examples of suitable nitrogen sources include, but are not limited to: ammonia, including ammonia gas and aqueous ammonia; ammonium salts of inorganic or organic acids, such as ammonium chloride, ammonium phosphate, ammonium sulfate and ammonium acetate; and other nitrogen-containing substances, including meat extract, peptone, corn steep liquor, casein hydrolysate, soybean cake hydrolysate and yeast extract.

[0106] Some amino acids present individually in minimal salts media may not be utilized well by bacteria as carbon sources. Each species of bacterium differs in their ability to utilize each natural amino acid. Amino acids that are not utilized individually may be utilized well in the presence of other amino acids, for instance serine may be used as a carbon source only if glycine, leucine, isoleucine and valine are present [25]. In rich media, such as synthetic amino acid mixes, several amino acids may be utilized preferentially and consumed before other amino acids. Serine, proline, glycine, aspartate, threonine, glutamate and alanine may be completely removed from a mix of the 16 amino acids present in caseamino acids, a popular media constituent, while the others are utilized more slowly and incompletely [26]. Similar results are obtained in tryptone broth [27]. If it is desirable to re-utilize amino acids accumulated as end metabolites, then the amino acid may be preferably serine, proline, glycine, aspartate, threonine, glutamate or alanine, and the medium may contain the additional amino acids needed to stimulate its use as a carbon source. The medium may also contain a protein hydrolyzate including, but not limited to, tryptones, caseamino acids and soy hydrolyzates.

EXAMPLE 1

Production of Reduced-Genomc Bacteria

[0107] The strains of reduced-genome bacteria listed in Table 2 were produced using scarless genome reduction methods discussed in copending U.S. Patent Application No. 10/057,582 and [28], which are incorporated herein by reference. MDS12, was constructed by removing the 12 largest K-islands, reducing the genome length by 8.1% and eliminating 24 of the 44 IS elements. In MDS40 more than 14% of the parent genome has beencumulatively removed, without compromising its ability to survive and grow at least as well as the standard wild-type strain MG1655. In MDS40 all the structural and regulatory genes for flagella have been removed. Flagella consume large amounts of energy and serve no useful purpose in the fermentation environment. Moreover, flagella are highly antigenic and may contaminate product preparations. The *tonA* (*fhuA*) gene was also deleted to provide phage resistance. In MDS41-R13, two IS1, one IS2 and one IS5, three of which had "hopped" during strain construction, were deleted, to create a strain completely free of ISs.

Table 2 - Reduced Genome Bacteria

Strain	# of deletions	% deleted	Genome size	DNA deleted
K-12 MG1655	0	0	4,639,221	0
MDS12	12	8.1	4,263,041	376,180
MDS39	39	14.1	3,984,362	654,859
MDS40	40	14.2	3,978,398	660,823
MDS41-R13	41	14.3	3,976,961	662,260

EXAMPLE 2

Growth Characteristics of MDS Strains

[0108] Growth of MDS41-R13, MG1655 and W3110 in batch culture was limited to about OD_{600} of 4.5 (Figure 23). At the cessation of growth, the pH of the media was about 4.5 due to excretion of acids. In contrast, MDS41-R13, MG1655 and W3110 grew in batch culture in Terrific Broth with 4% glycerol as the carbon source, with a doubling time of about 32 minutes (μ=1.3) to reach a much higher cell density (OD_{600} of approximately 16). This suggests overflow metabolism is limiting growth, since growth on glycerol is known to produce much less overflow metabolism than growth on glucose.

[0109] MDS41-R13 grew exponentially with doubling time of about 36 minutes (μ=1.2) in a batch fermentation experiment in an instrumented 17 liter laboratory fermenter in minimal salts with 2% glucose as the carbon source. Growth slowed down at an OD_{600} of approximately 4.2, despite control of pH with ammonium hydroxide (data not shown). In contrast, MDS40 (carrying the expression plasmid pPROEx-Cat, but un-induced) was still growing strongly at an OD_{600} of 23 in a fed-batch experiment undertaken with in instrumented, controlled laboratory fermenter (Figure 23A). In a separate experiment, MDS40 grew to an OD_{650} of 75 (approximately 30g dry cell mass per liter) at which point the fermenter was not able to provide sufficient oxygen to enable growth to continue. The exponential glucose feed was designed to maintain μ = 0.14 (a doubling time of almost 5 hours). Although the experiments were not completely comparable, due to strain differences, this is indicative that growth was limited in the batch culture by overflow metabolism.

[0110] MDS41-R13 grew at least as well as K-12 wild-type strains MG1655 and W3110 (both used widely in industry) in shake flasks in an industrial process medium used to produce tonnage-quantities of a specialty chemical. The medium contained 2% corn steep liquor and ammonium sulfate (as nitrogen sources) and 8.4% dextrose (as carbon source) and was buffered

to an initial pH of 6.8 with 80mM Bis-Tris (Figure 23B). In another experiment (Figure 23C), growth was measured in media with two different initial pHs – pH 6.8, as above; and pH 6.2, buffered with 50mM Bis-Tris. At the indicated times (arrows), when growth started to slow down, ammonium hydroxide solution was added to restore the pH to the original value. Growth was not nitrogen limited. The results suggest that growth was initially pH limited, and then limited by an unknown factor, most likely overflow metabolites. W3110 behaved identically (data not shown).

[0111] Figure 23D shows the ability of MDS40 to produce a model recombinant protein. The protein chloramphenicol acetyl transferase (CAT) was expressed from plasmid pPROEx-CAT (InVitrogen). Two cultures each of MDS40 and MG1655 containing the plasmid were grown in parallel in shake flasks in Korz minimal salts medium. Two cultures were induced with IPTG at 12 hours. The growth of all four cultures followed an almost identical, classical course. Both MDS40 and MG1655 produced CAT from the expression plasmids with similar kinetics following induction, however, MDS40 produced significantly higher specific activity overall.

EXAMPLE 3

Production of Phosphotransacetylase (pta) Deletion Strain

[0112] The *pta* gene was deleted in strain MG1655 using the gene gorging method as described in copending U.S. Patent Application No. 10/057,582, which is incorporated herein by reference. 500bp sections of genomic DNA upstream and downstream of the gene were PCR amplified and then fused, to create a synthetic deletion, precisely deleting the open reading frame. This DNA fragment was cloned into an InVitrogen Topo vector and then used for gene gorging, replacing the gene and flanking regions by homologous recombination. The desired mutant BTL5 was obtained by screening for poor anaerobic growth on agar plates. MG1655 and BTL5 were grown at 37°C in MOPS-minimal medium [29] containing 0.4% glucose in baffled Erlenmeyer flasks in an orbital shaker. Optical density at 650nM was measured to monitor cell growth. Metabolism was monitored by measuring the concentration of glucose in the medium and the pH of the medium. Acetate in the medium was measured, after removing the cells by centrifugation, using an enzymatic assay (Roche/R-biopharm). The results are shown in Figure 24 MG1655 produced and excreted into the medium copious amounts of acetate, reaching a concentration of approximately 5 mM after approximately 300 minutes of incubation. At this point the culture

entered stationary phase as determined by cessation of the increase of OD_{650}. The acetate was re-utilized during stationary phase, accounting for the gradual decrease in acetate from about 300 minutes on. BTL5 produced much less acetate than the parent strain MG1655, reaching a maximum concentration of slightly more than 1 mN by the end of the experiment.

[0113] The alcohol dehydrogenase gene (*adhE*) was then deleted to prevent ethanol production. The resulting mutant was screened by PCR to produce strain BTL3 (*pta adhE* double deletion). An advantage of gene gorging is that the designed modification is produced at a high frequency, making it efficient to simply screen for it using PCR. As expected, the double mutant strain excreted very little acetate in an anaerobic shake flask culture in M9 glucose-minimal salts medium (Figure 24). Pyruvate, lactate and malate accumulated in the medium, very similar to literature reports of *pta* mutants.

[0114] A group of additional mutants was prepared in which the phosphotransacetylase (*pta*) gene was deleted by Tn5 insertion. The *pta* or the *eutI* in the MG1655-pta, MG1655-eutI, MDS42-pta, MDS42-eutI, MG1655-pta-adhE and MDS42-pta-adhE strains was deleted by insertion mutation while the *adhE* was deleted by gene gorging (Herring et al., 2003). In order to determine the doubling times, all strains were grown at 37°C with shaking at 175 rpm in shake flasks containing 50 ml MOPS minimal medium supplemented with 0.2% glucose. Cell number was based on OD_{600} with samples being taken every 12 min.

Table 3

Strain	Doubling time (min)
MG1655	67.0
MG1655-pta	74.4
MG1655-eutI	67.1
MG1655-pta, adhE	68.2
MDS42	69.4
MDS42-pta	72.3
MDS42-eutI	69.2
MDS42-pta, adhE	73.9

[0115] As a comparison, MDS42 and MDS42-pta were evaluated for their performance in cell growth, by-product formation and also protein production using CAT as a reporter. Fermentation was carried out in a 2-L BIOFLO 110 Fermentor (New Brunswick Scientific) with a 1.5-L working volume. A modified Korz medium was chosen because this was a well-documented industrial fermentation medium to achieve "high-cell-density-culture" (HCDC) of *E. coli* cells,

which is the basis for recombinant protein production (Korz et al., 1995; Gill et al, 2001). The strains were first grown in a batch medium containing 5 g/L glucose, 8 g/L KH_2PO_4, 0.4 g/L $MgSO_4$ and trace elements. Then the cultures were shifted to a fed-batch mode, which is commonly used in industrial *E. coli* fermentation processes. The feed medium contains 481 g/L glucose, 4 g/L $MgSO_4$ and 1.5x trace elements as described in Korz et al. (1995). The seed culture for the batch fermentation was started from a frozen glycerol stock to inoculate a 200-ml batch medium containing 20 g/L glucose in a 1-L flask. The cells were then grown to 0.75 OD_{600} unit and pumped in to inoculate 750-ml batch medium (5% v/v). The pH value of the medium was controlled at 6.75 by NH_4OH during fermentation and the temperature was controlled at 37°C. Air was sparged into the vessels at a constant rate and the dissolved oxygen (DO) rate was maintained at 30% of saturation by varying the agitation rate from 300 rpm to 1200 rpm. The batch phase was operated for 10 h to allow the cells to completely consume the glucose in the medium. Feeding was started during the fed-batch phase (for 10 h) at an exponential rate to control cell growth and minimize acetate accumulation (Akesson et al., 1999; Delisa et al., 1999). To test protein production, a pPROEx-CAT (Invitrogen) plasmid was transformed and expressed in the MDS strains. Ampicillin was added to the culture to maintain the pPROEx-CAT plasmid. After the cells were shifted to the fed-batch phase, 1 mM IPTG was added to induce CAT protein production. A CAT assay was performed to determine the specific CAT activity. Acetate accumulation was determined by using an assay kit (Roche Biochemicals). The MDS42-pta strain produced less biomass (42.3 OD vs 69 OD), much less acetate formation (1.8 g/L vs 4.2 g/L), and higher CAT specificity (743 U/mg vs 620 U/mg) (Compare Figure 25 to Figure 26).

EXAMPLE 4

Production of Threonine-Producing Strain

[0116] A strain for producing threonine is produced by introducing a suitable threonine operon construct and making other genetic modifications. The expression system for the threonine operon ideally includes a strong promoter and is inducible to enable the activity of the threonine pathway to be controlled. The expression system is also convertible to constitutive operation for routine industrial use to avoid the use of inducers and is compatible with a plasmid-based

recombinant protein expression system. Tac/IPTG and tet/anhydrotetracycline may be used as promoters/inducers for threonine and recombinant protein expression, respectively.

[0117] The construction of the strain is designed to achieve (1) threonine production, (2) good genetic stability, and (3) minimal effects on growth rate. A threonine operon with a feedback-resistant *thrA* gene may be transferred from strain ATCC21277 [30] into MDS41E by gene gorging [31]. The mutant *thrA* gene and an appropriate segment of upstream and downstream genomic DNA are amplified using high fidelity PCR and cloned into a pTOPO donor plasmid, flanked by I-sceI sites and electroporated with pACBSR into MDS41E. Co-transformants are selected using antibiotic resistance markers. The I-sceI homing endonuclease and lambda red recombination system of pACBSR are induced with arabinose, causing the mutant *thrA* gene and flanking homology regions to be excised from pTOPO and integrated into the chromosome of MDS41E by homologous recombination. After curing the plasmids, the desired integrants are identified by screening clones using PCR. Alternatively, MDS41E could first be made *threA-* (threonine requiring on minimal medium), allowing selection of the desired integrants on media lacking threonine. Suitable mutants could also be produced by exposing the bacteria to a mutagenic agent such as nitrosoguanidine and selecting mutants that are resistant to α-amino-β-hydroxyvaleric acid, or the operon could be transferred into the *thrA-* strain using P1 transduction.

[0118] A suitable inducible, strong promoter, such as tac, is introduced upstream of *thrA* using gene gorging, eliminating the attenuator region and with it the unwanted feedback repression. The operon is inducible with IPTG in glucose-containing media, is controllable by changing the IPTG concentration, and can be made constitutive by inactivating the chromosomal repressor gene *lacI*. The desired integrants are screened using PCR and/or inducible threonine production. Threonine is measured using a bioassay, based on a *thrA-* strain (threonine auxotroph). If necessary, the threonine dehydrogenase gene is deleted using the gene gorging method or the inactivated gene is introduced from strain CGSC6945. The resulting strain excretes threonine to high levels (>60 mM). Furthermore, the production rate is controllable by varying the inducer concentration.

[0119] A series of L-threonine-producing hosts based on the MG1655 or the MDS42 background were generated (Table 4). The operon encoding for L-threonine biosynthesis, *thrABC*, was cloned by PCR. The *thrA* gene was mutated (*thrA**) so the feedback regulation of

the operon is eliminated. The entire operon was driven by an inducible *Ptac* promoter. This *Ptac-thrA*BC* construct was linked to a kanamycin (Kan) resistance marker for selection and introduced into each background as the P strain. The *lacI* gene in P was then disrupted by the *tetA* gene, which codes for tetracycline resistance. Deletion of *lacI* allows constitutive expression of the *thrA*BC* operon from the *Ptac* promoter. The new host in MG1655 or MDS42 was named as I. In order to reduce threonine degradation, a *tdh* gene (threonine dehydrogenase) was disrupted by the *CAT* gene in I. The resultant strain in each background was named the T strain. To increase product secretion to the production medium, an *rhtA23* gene that codes for L-threonine export, was integrated into the chromosome of the T strain to make the F strain in MG1655 or MDS42. In other attempts, the *rhtA23* gene was introduced in vector p177CR (low copy-number) or pTOPOCR (high-copy number). Each plasmid was then transformed into the F strain to obtain different level of expression of the *rhtA23* gene (F[p177CR] or F[pTOPOCR]).

Table 4

Strain	Genotype
MG1655 or MDS42	
P	Ptac-thrA*BC
I	*Ptac-thrA*BC, lacI::tetA*
T	*Ptac-thrA*BC, lacI::tetA, tdh::CAT*
F	*Ptac-thrA*BC, lacI::tetA, tdh::CAT, rhtA23(1 copy)*
F[p177CR]	*Ptac-thrA*BC, lacI::tetA, tdh::CAT, rhtA23[p177CR], low-copy*
F[pTOPOCR]	*Ptac-thrA*BC, lacI::tetA, tdh::CAT, rhtA23[pTOPOCR], high- copy*
F-Δ*tdh*	*Ptac-thrA*BC, lacI::tetA, Δtdh, rhtA23*
F-Δ*tdcC*	*Ptac-thrA*BC, lacI::tetA, Δtdh, ΔtdcC, rhtA23*
F-Δ*lacI*	*Ptac-thrA*BC, Δ lacI, Δtdh, ΔtdcC, rhtA23*
F-Δ*pta*	*Ptac-thrA*BC, Δ lacI, Δtdh, Δpta, rhtA23*

[0120] To further improve threonine production, *tdh* was deleted by markless deletion method to create MDS42F-Δ*tdh*. In this background, a *tdcC* gene that codes for threonine influx was deleted by markless deletion so the threonine produced can not be transported back to the cells once it is secreted to the medium. The resultant strain was named as MDS42F-Δ*tdcC*. In order to obtain the *tetA* marker back, the *lacI* gene was deleted by markless deletion in MDS42F-Δ*tdcC* to make MDS42F-Δ*lacI*.

[0121] To reduce acetate formation and further improve threonine production in the MDS42F-Δ*lacI* strain, the gene coding for phosphotransacetylase (*pta*) was deleted to create MDS42-F-Δ*pta*. The production of L-threonine was evaluated in several of the strains (Figure 27). As indicated, MDS42 strains with the *Ptac-thrA*BC* construct and disrupted *lacI* and *tdh* genes outperformed their patental strain (MG1655) in L-threonine production.

EXAMPLE 5

Production of Recombinant Protein in Threonine-Producing Strain

[0122] Growth and recombinant protein production would be evaluated in the modified strains of MDS41E (or an appropriate related strain), as a function of the amount of threonine produced, in standard shake flask cultures and in synchronized well-controlled fed-batch fermentations in an instrumented, computer-controlled laboratory fermenter. These strains would be compared with the parent strain.

[0123] A suitable expression plasmid carrying the chloramphenicol O-acetlytransferase gene under the control of an inducible promoter compatible with the threonine operon promoter (for example the tet promoter, derepressible with anhydrotetracycline, if tac is used for the threonine operon) is constructed, and transformed using electroporation into the threonine pathway modified strain of [0114] and the parent strain.

[0124] A 3L fed-batch fermenter is inoculated with an overnight shake flask culture using an amino acid free minimal medium such as that described by Korz [32] or in standard MOPS-minimal medium [29] or M9 medium. Culture parameters are monitored including cell density, glucose concentration, pH, temperature, and dissolved oxygen. Cell yields, acetate production and recombinant protein productivity are evaluated, the latter by standard enzymatic assays. Sterile antifoam is added as needed. Cultures are agitated between 300 and 1200 rpm and airflow rate varied to control the dissolved oxygen (DO) level to 25%. Sterile NH_4OH is added to control pH on-line. Cells are cultured in minimal medium with glucose, until the glucose was consumed. A medium containing glucose and trace salts is then fed to maintain the desired growth rate. Expression of recombinant protein is induced with anhydrotetracycline when the cell density reaches approximately 70% of the maximum level. The total dry cell weight and the recombinant protein content are determined. The glucose concentration are measured using a glucose meter (LifeScan One Touch Profile). Acetate is determined enzymatically (Roche/R-

biopharm kit). The optical density is measured with a Spectronic 20 Genosys spectrophotometer at 600 nm or 650nm on-line using a flowcell or off-line by removing samples to a cuvette. All experiments are conducted in triplicate. At each time point, 30mL of culture is harvested for total protein, recombinant protein activity, and if appropriate, Western blot assays. Total protein and specific CAT activity or other protein activity are determined. Western blots are used to detect recombinant proteins and determine protein integrity. The blots are scanned and quantified using a densitometer (BioRad). Results indicate that threonine is produced and excreted into the medium greater than 100mM (11.9g/l) while retaining at least 75% of the volumetric productivity of biomass and recombinant protein production of the parent strain carrying the protein expression system.

[0125] A series of L-threonine-producing hosts based on the MG1655 or the MDS42 background were generated. The operon encoding for L-threonine biosynthesis, *thrABC*, was cloned by PCR. The *thrA* gene was mutated (*thrA**) so the feedback regulation of the operon was eliminated. The entire operon was driven by an inducible *Ptac* promoter. This *Ptac-thrA*BC* construct was linked to a kanamycin (Kan) resistance marker for selection and introduced into each background as the P strain. The *lacI* gene in P was then disrupted by the *tetA* gene, which codes for tetracycline resistance. Deletion of *lacI* allows constitutive expression of the *thrA*BC* operon from the *Ptac* promoter. The new host in MG1655 or MDS42 was named as I. In order to reduce threonine degradation, a *tdh* gene (threonine dehydrogenase) was disrupted by the *CAT* gene in I. The resultant strain in each background was named the T strain. To increase product secretion to the production medium, an *rhtA23* gene that codes for L-threonine export, was integrated into the chromosome of the T strain to make the F strain in MG1655 or MDS42. In addition, the *rhtA23* gene was introduced in vector p177CR (low copy-number) or pTOPOCR (high-copy number). Each plasmid was then transformed into the F strain to obtain different level of expression of the *rhtA23* gene (F[p177CR] or F[pTOPOCR]).

[0126] To further improve threonine production, *tdh* was deleted by markless deletion method to create MDS42F-Δ*tdh*. In this background, a *tdcC* gene that codes for threonine influx were deleted by markless deletion so the threonine produced can not be transported back to the cells once it is secreted to the medium. The resultant strain was named as MDS42F-Δ*tdcC*. In order to obtain the *tetA* marker back, the *lacI* gene was deleted by markless deletion in MDS42F-Δ*tdcC* to make MDS42F-Δ*lacI*. To reduce acetate formation and further improve threonine production

in the MDS42F-Δ*lacI* strain, the gene coding for phosphotransacetylase (*pta*) was deleted to create MDS42-F-Δ*pta*.

[0127] The suitability of the MDS42Δtdh and MG1655 Δtdh strains *(Kan-Ptac-thrA*BC,lacI::tetA, Δtdh,rhtA23)* for recombinant protein expression was tested by expression of 2 genes form *Arabidopsis thaliana*. The MDS42 strain was used as a reference. The vector used to express these cloned genes is under the control of a T5 phage based promoter that is IPTG or lactose inducible. They are fused to an N-terminal His-tagged, maltose binding protein (MBP) and a TEV protease cleavage site is between the MBP and target protein. The expected molecular weight of the recombinant protein products are 72.3kDa and 64.7kDa.

[0128] Each of the genes was over-expressed using IPTG induction in TB media supplemented with 1 mM $MgSO_4$, 25 mM $(NH4)_2SO_4$, 50mM KH_2PO_4 and 25 mM Na_2 HPO_4, or an auto-induction employing a chemically defined media (Overnight Express System 2, EMD Biosciences). MDS42 cultures were supplemented with ampicillin (100 μg/ml) and MDS42Δtdh and MG1655 Δtdh were supplemented with ampicillin and kanamycin (100 μg/ml and 50 μg/ml, respectively). All cultures were grown at 37°C in 2 ml volumes using 17x100 mm tubes. In the case of IPTG induction, once the cultures reached OD_{600} nm 0.6-1, IPTG was added to a final concentration of 2 mM. Culture were grown at 37°C for additional 3h. Auto-induction cultures were grown at 37°C for 20h. Following induction, a 300 μl aliquot of each culture was harvested and cells lysed by addition of 2X SDS-PAGE gel loading buffer and 5 min incubation at 95°C. Equal volume (12 μl) of the extracts was analyzed by SDS-PAGE (4-20% gradient gel) and Coomassie blue-R250 staining (Figure 28).

EXAMPLE 6

Production of Acetate-Blocked Strains

[0129] The gene coding for phosphotransacetylase (*pta*) is precisely deleted from MDS41E, essentially as described in Example 3 above. The *ackA* gene is deleted in a similar manner as to the deletion of *pta*. An expression plasmid containing the recombinant chloramphenicol acetyltransferase (rCAT) gene under the control of the tet promoter is introduced by electroporation into the deletion strains.

[0130] The resulting Δ*pta* and Δ*pta*-Δ*ackA* strains are evaluated in shake flask cultures for growth characteristics (growth rate and maximum cell density), recombinant protein production

and acetate production. 25 ml cultures in 250ml baffled shake flasks are inoculated with cells from overnight cultures and grown with vigorous shaking in an orbital shaker to achieve maximum oxygenation. The medium is a minimal salts medium containing various concentrations of glucose.

[0131] In a first series of experiments, growth and acetate production of MDS41E Δ*pta*-Δ*ackA* is evaluated as a function of glucose concentration from 0.1% to 8%, representing a range of concentrations from below to above the critical concentrations [causing overflow metabolism in the wild-type strain, and compared with unmodified MDS41E. The concentration of acetate, pyruvate and other metabolites in the medium is measured. In a second series of experiments, rCAT expression is induced by addition of anhydrotetracycline (100μM to 200μM) in late exponential phase. Biomass production, recombinant protein production, and accumulation of acetate, pyruvate and other metabolites in the medium will be evaluated at appropriate time points.

[0132] The Δ*pta*-Δ*ackA* strain grows slower than unmodified MDS41-R13, due to the feedback inhibition of PTS-mediated glucose assimilation by the increased intracellular pyruvate concentration. The culture reaches a higher final cell density, at least in media containing glucose above the critical concentration, due to reduced growth inhibition by acetate. The Δ*pta*-Δ*ackA* strain shows greatly reduced accumulation of acetate. Pyruvate and/or other metabolites accumulates in the medium. The total amount of recombinant protein is higher in the Δ*pta*-Δ*ackA* strain, due to its longer growth and higher final cell density. In minimal salts media with glucose concentration above the critical concentration, the Δ*pta*-Δ*ackA* has a greater than 75% reduction of aerobic acetate production.

[0133] As previously discussed, *eutI* may contribute to acetate formation. We identified the *eutI* operon in the MG1655 genome. The *eutI* operon was removed from MG1655 by deleting from 2552150 to 2573897, which covers the entire *eutI* operon and other regions that are covered in other reduced genome bacteria. A comparison of the growth rate of strains with a deleted *pta* operon or *eutI* operon to a parent MDS42 strain is shown in Table 5.

Table 5

Strain	Operon name	Starting point	Ending point	Doubling time
MDS42	Starting strain			69.4 min
MDS42-X	*pta* operon	2411490	2416621	74.4 min
MDS42-Y	*eutI* operon	2552150	2573897	71.3 min

EXAMPLE 7

Production of an Acetate-Blocked Threonine Excretor

[0134] The *pta* and *ackA* genes are deleted, using the methods described above, in the threonine producing strain derived from MDS41E above. The rCAT expression plasmid is then electroporated into the modified strain. The strains are evaluated as described above, except that IPTG is added to the growth medium to induce and control the activity of the threonine pathway. Following the biomass production phase, at about 75% of maximal cell density, anhydrotetracycline is added to induce and control the recombinant protein (rCAT) production. The IPTG induced threonine producer-Δ*pta*-Δ*ackA* dual pathway-modified strain is compared with the uninduced strain, and with the strains with separate modifications in each pathway. Biomass production, recombinant protein production (CAT), dissolved oxygen (DO), and accumulation of acetate, pyruvate and other metabolites in the medium are evaluated at appropriate time points and with (1) varying glucose concentrations above and below the critical concentrations causing overflow metabolism in the wild-type strain (2) varying levels of induction of the threonine pathway and (3) varying levels of induction of recombinant protein production. The threonine concentration in the medium are measured, using the Dionex LC system. Key metabolites, including PEP, pyruvate and acetyl CoA are extracted from the cells at varying times using standard methods and assayed using methods based on the Dionex LC instrumentation, or enzymatic methods.

[0135] The modified strain produces much less acetate compared with the wild-type strain at glucose concentrations above the critical concentration, and also produces threonine. Compared to the individual pathway modified strains, the dual pathway modified strains: (1) produce more threonine, (2) produce much less acetate (3) excrete reduced amounts of other overflow metabolites such as pyruvate and glutamate into the medium, (4) grow faster than the acetate

pathway mutant (5) achieve a higher final cell density than MDS41E on high glucose media, and (6) produce recombinant protein at a higher rate and in greater final quantity than in MDS41E.

EXAMPLE 8

Maximizaion of the Volumetric Productivity of Biomass and Recombinant Protein Production

[0136] Depending on the desired results, the flux through the threonine pathway is either decreased or increased. The former would be straightforward. Increasing the flux may require further modifications to redirect overflow metabolites towards oxaloacetate (anaplerotic pathways). It may also be necessary to improve the supply of building blocks for protein synthesis, and this may be achieved the same way. Full optimization might require more than one iteration of the design-create-evaluate-interpret cycle, as indicated by the reverse-direction dotted arrows in the Gannt chart (Figure 29).

[0137] The following modifications may be made alone or in combination to achieve greater flux towards threonine or accumulation outside the cell: (1) delete or inactivate the threonine dehydrogenase (*tdh*) to prevent breakdown of threonine; (2) delete or inactivate the LIVI pathway; (*livJ*); (3) delete or inactivate catabolic threonine deaminase; (*tdc*); (4) increase the expression of the threonine operon, by optimizing the promoter construct, or putting additional copies of the synthetic, inducible threonine operon into the chromosome; (5) mutate the threonine exporter *rhtA*; (for example, introduce the mutation rhtA23), to increase the excretion of threonine; (6) overexpress the aspartate semialdehyde dehydrogenase (*asd*), the second enzyme in the threonine pathway, thereby making the expression of the threonine pathway constitutive, to avoid the need for induction by inactivating the chromosomal *lacI* gene; (7) increase the expression of phosphoenolpyruvate carboxylase (*ppc*) (for example, by putting it under the control of a heterologous promoter), to increase the flux of pyruvate to oxaloacetate, thereby increasing the supply of precursors for threonine production (via aspartate) and also replenishing the TCA cycle for synthesis of other metabolites; (8) modify phosphoenolpyruvate carboxylase (*ppc*) to reduce feedback inhibition by aspartate; and (9) introduce and overexpress a heterologous pyruvate carboxylase (*pyc*) gene.

[0138] The latter three modifications may have an anaplerotic effect, replenishing the building blocks via the TCA cycle.

EXAMPLE 9

Manipulation of a Regulator of Acetate Formation

[0139] Phue and Shiloach (2004) compared the glyoxylate shunt enzyme levels of a low-acetate producer (BL21, a B strain) and a high-acetate producer (JM109, a K strain). BL21 is a recognized low acetate producer that has been used for recombinant protein production. The glyoxylate pathway in BL21 is constitutively active during glucose fermentation due to a fairly low level of isocitrate lyase repressor (ICLR). In contrast, the ICLR level is very high in JM109 cells when grown under the same conditions. ICLR negatively regulates the *aceABK* orperon, which contains isocitrate lyase (*aceA*), malate synthase (*aceB*) and isocitrate dehydrogenase (*aceK*). A deletion mutant of *iclR* was created via gene gorging.

[0140] To study ICLR, a deletion mutant of *iclR* was created by gene gorging. We evaluated this mutant along with MG1655 in shake-flask fermentation. The strains were first grown on MOPS minimal medium at 30 °C for 2 days to get single colonies. Then a single colony was used to inoculate 10 ml of the Korz medium containing 20 g/L glucose in a 25-ml baffled flask. The cells were shaken at 330 rpm for overnight and then used to start the fermentation experiments. Fermentation was carried out at 30 °C with a starting pH of 6.9. The pre-cultured cells were grown in a 100-ml fresh Korz batch medium containing 5 g/L glucose at 30°C with shaking at 330 rpm. The cells were then fed with 1 ml of the feeding medium every h, and the pH was adjusted to maintain 6.2-6.7 every h manually.

[0141] Deletion of *iclR* in MG1655 led to a 25% higher cell growth in the mutant compared to the parent (Figure 30). The mutant also produced less acetate at the end of the fermentation, 0.29 g/L vs 0.39 g/L from MG1655. This suggests that the MG1655-based strains, like the MDS strains, may have improved growth by increasing glyoxylate shunt during growth and fermentation on glucose.

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CLAIMS

1. A strain of a microorganism exhibiting the following properties in a growth medium comprising glucose:

(a) flux of production of a toxic metabolite reduced by at least 50% compared to a wild type microorganism;

(b) flux to one or more alternative metabolites of at least 10% of the flux of glucose into glycolysis; and

(c) growth rate of at least 50% of said wild type microorganism,

wherein the toxic metabolite is acetate.

2. The method of claim 1 wherein the concentration of glucose in the medium is at least 0.4%.

3. The method of claim 1 wherein the toxic metabolite is acetate.

4. The method of claim 3 wherein the strain comprises an inactivated *pta* gene.

5. The method of claim 4 wherein the strain further comprises an inactivated *eutI* gene.

6. The method of claim 1 wherein the alternative metabolite is an L-amino acid selected from the group consisting of L-alanine, L-valine, L-leucine, L-aspartate, L-asparagine, L-lysine, L-methionine, L-threonine, L isoleucine, L-glutamate, L-glutamine, L-proline, L-arginine, L-tryptophan, L-tyrosine, L-phenylalanine, L-serine, L-glycine, L-cysteine, and L-histidine.

7. The method of claim 6 wherein the alternative metabolite is L-threonine and the strain comprises a *Ptac-thrA*BC* construct and an inactivated *lacI* and *tdh* gene.

8. The method of claim 6 wherein the strain further comprises a heterologous gene operatively linked to a promoter.

9. The method of claim 1 wherein the alternative metabolite is selected from the group consisting of glycerol, glycogen, trehalose, saccharides, acetaldehyde, ethanol, lactate, formate, oxaloacetate, citrate, succinate and succinyl-CoA.

FIGURE 1

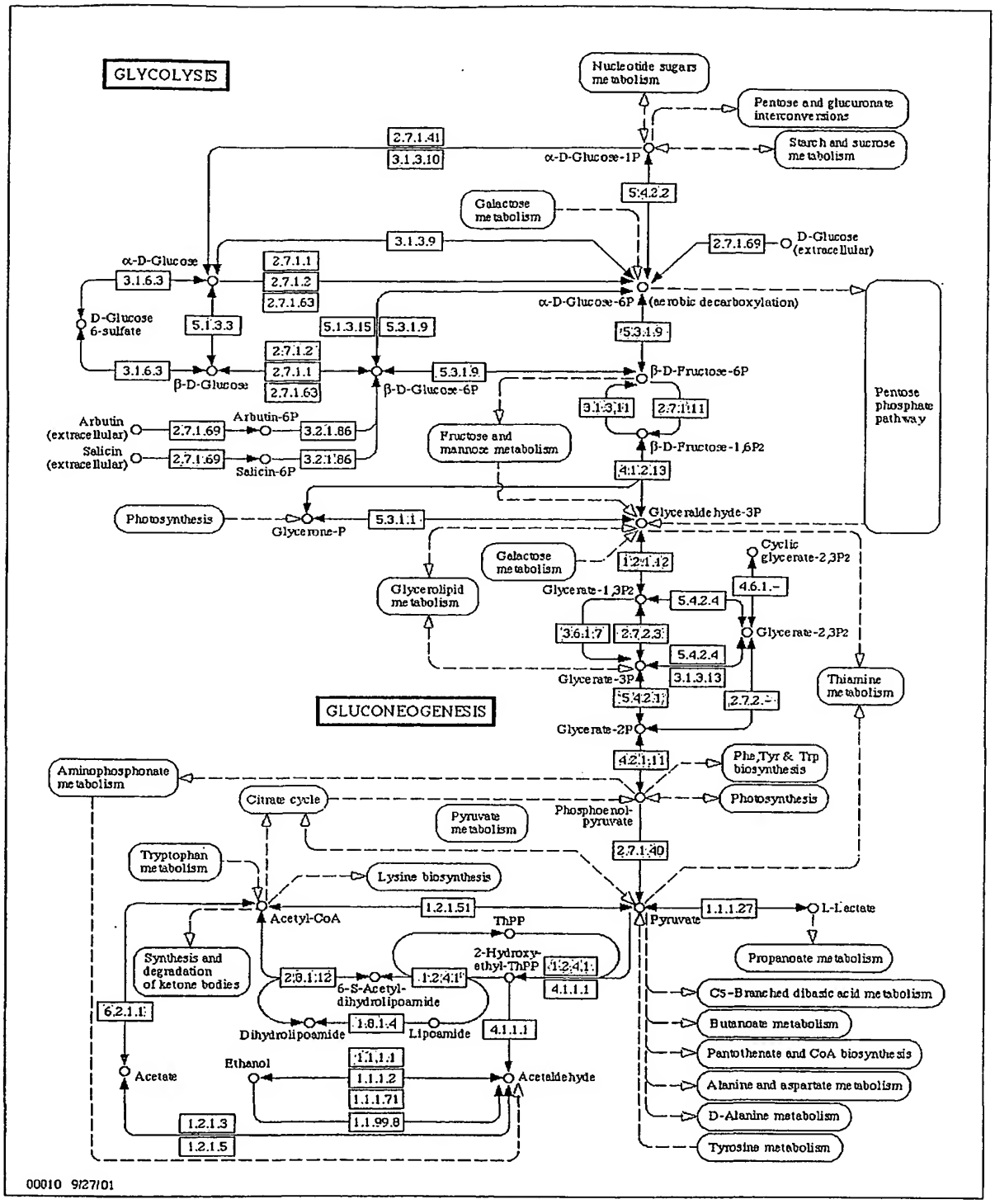
GLYCOLYSIS
GLUCONEOGENESIS
Nucleotide sugars metabolism
Pentose and glucuronate interconversions
Starch and sucrose metabolism
α-D-Glucose-1P
2.7.1.41
3.1.3.10
5.4.2.2
Galactose metabolism
3.1.3.9
2.7.1.69
D-Glucose (extracellular)
α-D-Glucose
3.1.6.3
2.7.1.1
2.7.1.2
2.7.1.63
α-D-Glucose-6P
(aerobic decarboxylation)
D-Glucose 6-sulfate
5.1.3.3
5.1.3.15
5.3.1.9
β-D-Fructose-6P
β-D-Glucose
β-D-Glucose-6P
Pentose phosphate pathway
Arbutin (extracellular)
Arbutin-6P
3.2.1.86
Salicin (extracellular)
Salicin-6P
Fructose and mannose metabolism
2.7.1.11
β-D-Fructose-1,6P2
4.1.2.13
Photosynthesis
5.3.1.1
Glycerone-P
Glyceraldehyde-3P
Cyclic glycerate-2,3P2
1.2.1.12
Glycerolipid metabolism
Glycerate-1,3P2
4.6.1.-
5.4.2.4
3.6.1.7
2.7.2.3
Glycerate-2,3P2
Glycerate-3P
3.1.3.13
Thiamine metabolism
5.4.2.1
2.7.2.-
Glycerate-2P
4.2.1.11
Aminophosphonate metabolism
Phe,Tyr & Trp biosynthesis
Citrate cycle
Photosynthesis
Pyruvate metabolism
Phosphoenol-pyruvate
2.7.1.40
Tryptophan metabolism
Lysine biosynthesis
Acetyl-CoA
1.2.1.51
ThPP
Pyruvate
1.1.1.27
L-Lactate
2-Hydroxy-ethyl-ThPP
Propanoate metabolism
Synthesis and degradation of ketone bodies
2.3.1.12
1.2.4.1
1.2.4.1
4.1.1.1
6-S-Acetyl-dihydrolipoamide
C5-Branched dibasic acid metabolism
6.2.1.1
Dihydrolipoamide
1.8.1.4
Lipoamide
4.1.1.1
Butanoate metabolism
Pantothenate and CoA biosynthesis
Ethanol
1.1.1.1
1.1.1.2
1.1.1.71
1.1.99.8
Acetaldehyde
Acetate
Alanine and aspartate metabolism
D-Alanine metabolism
1.2.1.3
1.2.1.5
Tyrosine metabolism
00010 9/27/01

FIGURE 2

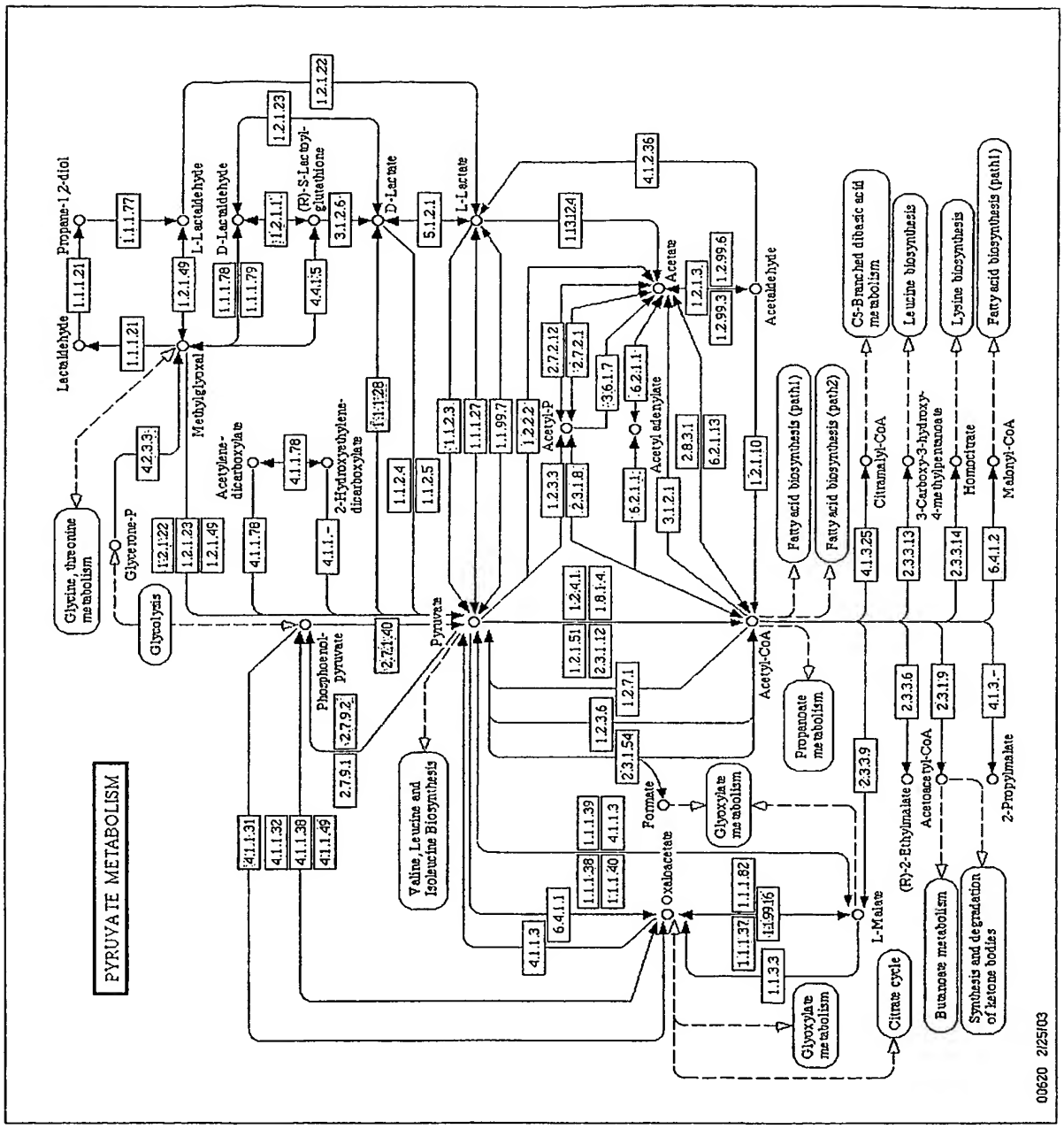
PYRUVATE METABOLISM
Propane-1,2-diol
Lactaldehyde
Methylglyoxal
L-Lactaldehyde
D-Lactaldehyde
(R)-S-Lactoyl-glutathione
D-Lactate
L-Lactate
Acetol
Acetaldehyde
Acetate
Acetyl-CoA
Acetyl adenylate
Acetyl-P
Glycine, threonine metabolism
Glycerone-P
Glycolysis
Acetylene-dicarboxylate
2-Hydroxyethylene-dicarboxylate
Phosphenol-pyruvate
Pyruvate
Valine, Leucine and Isoleucine Biosynthesis
Formate
Oxaloacetate
L-Malate
Glyoxylate metabolism
Propanoate metabolism
Fatty acid biosynthesis (path1)
Fatty acid biosynthesis (path2)
C5-Branched dibasic acid metabolism
Citramalyl-CoA
3-Carboxy-3-hydroxy-4-methylpentanoate
Homocitrate
Malonyl-CoA
Leucine biosynthesis
Lysine biosynthesis
Fatty acid biosynthesis (path1)
(R)-2-Ethylmalate
Acetoacetyl-CoA
2-Propylmalate
Citrate cycle
Butanoate metabolism
Synthesis and degradation of ketone bodies
00620 2/25/03

FIGURE 3

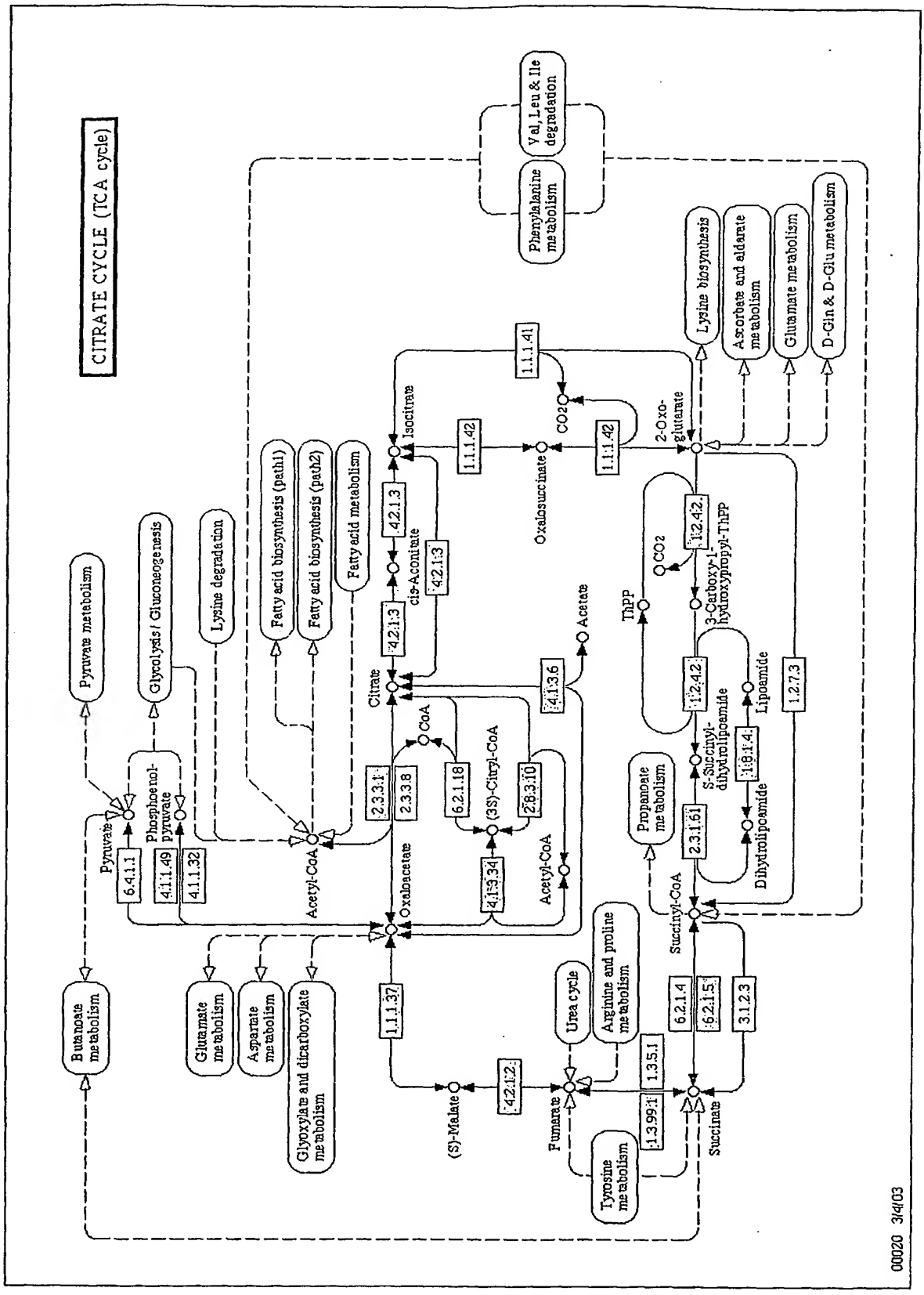
CITRATE CYCLE (TCA cycle)
Pyruvate metabolism
Glycolysis/ Gluconeogenesis
Lysine degradation
Fatty acid biosynthesis (path1)
Fatty acid biosynthesis (path2)
Fatty acid metabolism
Phenylalanine metabolism
Val, Leu & Ile degradation
Lysine biosynthesis
Ascorbate and aldarate metabolism
Glutamate metabolism
D-Gln & D-Glu metabolism
Butanoate metabolism
Glutamate metabolism
Aspartate metabolism
Glyoxylate and dicarboxylate metabolism
Urea cycle
Arginine and proline metabolism
Propanoate metabolism
Tyrosine metabolism
Pyruvate
Phosphoenolpyruvate
Acetyl-CoA
Oxaloacetate
Citrate
cis-Aconitate
Isocitrate
Oxalosuccinate
2-Oxo-glutarate
CO2
CoA
Acetate
ThPP
3-Carboxy-1-hydroxypropyl-ThPP
S-Succinyldihydrolipoamide
Lipoamide
Dihydrolipoamide
Succinyl-CoA
(3S)-Citryl-CoA
(S)-Malate
Fumarate
Succinate
6.4.1.1
4.1.1.49
4.1.1.32
2.3.3.1
2.3.3.8
6.2.1.18
2.8.3.10
4.1.3.6
4.2.1.3
4.2.1.3
4.1.3.34
1.1.1.37
4.2.1.2
1.3.5.1
1.3.99.1
6.2.1.4
6.2.1.5
3.1.2.3
1.1.1.41
1.1.1.42
1.2.4.2
1.2.4.2
2.3.1.61
1.8.1.4
1.2.7.3
00020 3/4/03

FIGURE 4

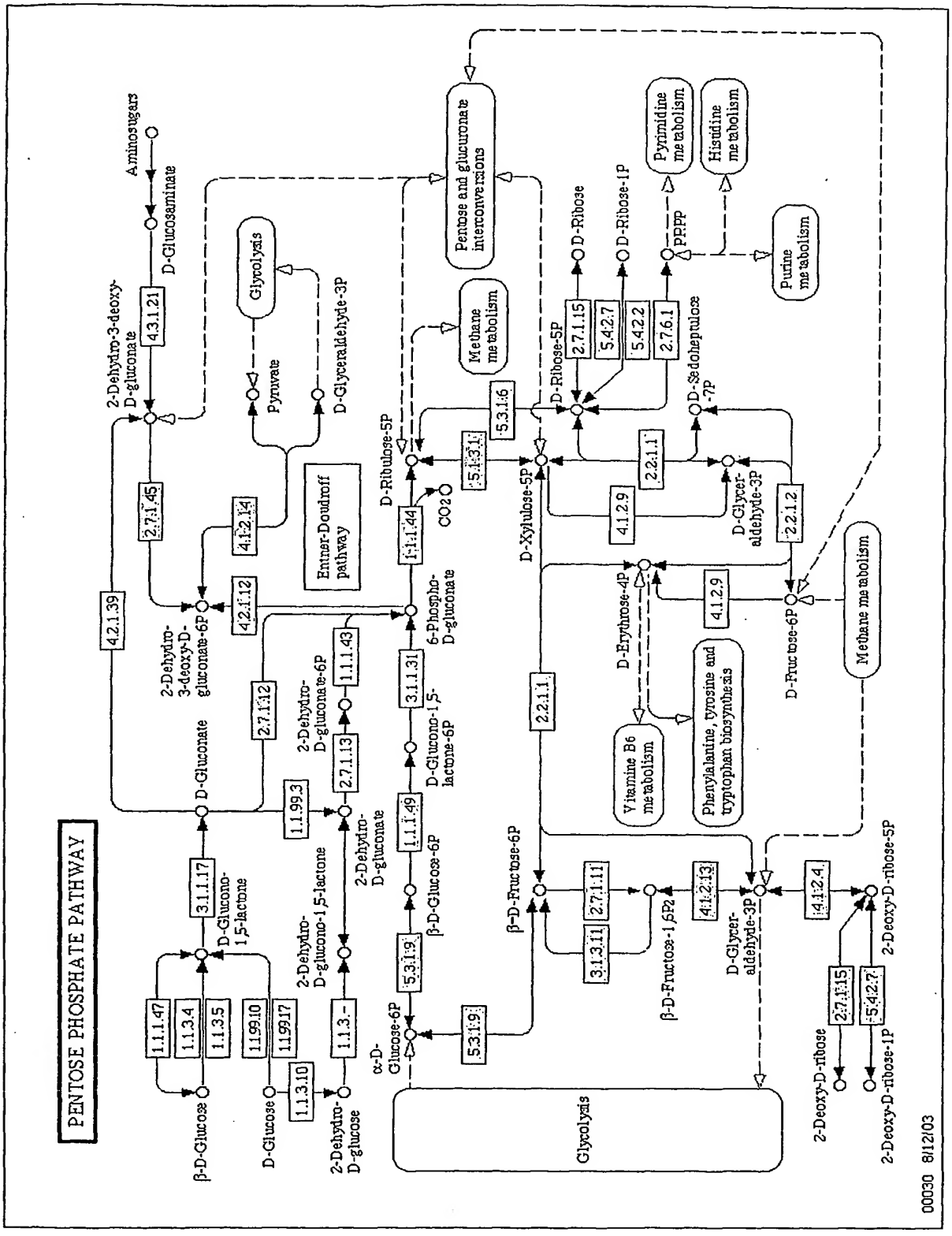
PENTOSE PHOSPHATE PATHWAY
Aminosugars
D-Glucosaminate
2-Dehydro-3-deoxy-D-gluconate
4.3.1.21
2.7.1.45
2-Dehydro-3-deoxy-D-gluconate-6P
4.2.1.39
D-Gluconate
2.7.1.12
2-Dehydro-D-gluconate-6P
1.1.1.43
2-Dehydro-D-gluconate
1.1.99.3
2.7.1.13
1.1.1.49
6-Phospho-D-gluconate
3.1.1.31
D-Glucono-1,5-lactone-6P
D-Glucono-1,5-lactone
3.1.1.17
β-D-Glucose-6P
5.3.1.9
α-D-Glucose-6P
1.1.3.-
2-Dehydro-D-glucose
1.1.3.10
β-D-Glucose
1.1.1.47
1.1.3.4
1.1.3.5
1.1.99.10
1.1.99.17
4.2.1.12
4.1.2.14
Entner-Doudnoff pathway
Glycolysis
Pyruvate
D-Glyceraldehyde-3P
Methane metabolism
Pentose and glucuronate interconversions
D-Ribulose-5P
1.1.1.44
CO2
5.1.3.1
5.3.1.6
D-Ribose-5P
D-Xylulose-5P
2.2.1.1
2.2.1.2
4.1.2.9
D-Glycer-aldehyde-3P
D-Sedoheptulose-7P
2.7.1.15
5.4.2.7
5.4.2.2
2.7.6.1
D-Ribose
D-Ribose-1P
PRPP
Pyrimidine metabolism
Histidine metabolism
Purine metabolism
D-Erythrose-4P
Vitamine B6 metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
D-Fructose-6P
Methane metabolism
β-D-Fructose-6P
2.7.1.11
3.1.3.11
β-D-Fructose-1,6P2
4.1.2.13
D-Glycer-aldehyde-3P
4.1.2.4
2-Deoxy-D-ribose-5P
2-Deoxy-D-ribose
2.7.1.15
5.4.2.7
2-Deoxy-D-ribose-1P
Glycolysis
00030 8/12/03

FIGURE 5

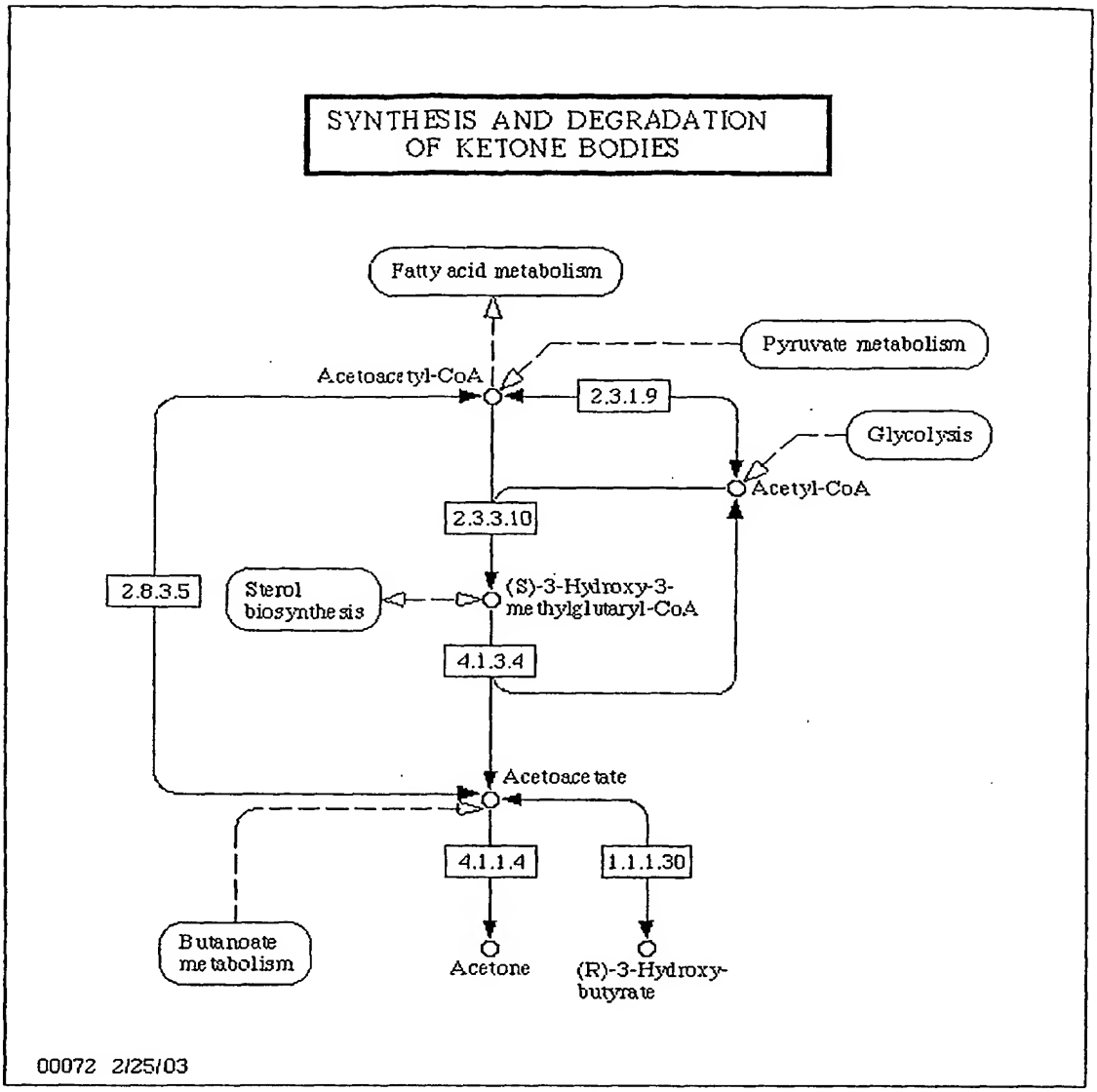
SYNTHESIS AND DEGRADATION OF KETONE BODIES
Fatty acid metabolism
Pyruvate metabolism
Acetoacetyl-CoA
2.3.1.9
Glycolysis
Acetyl-CoA
2.3.3.10
2.8.3.5
Sterol biosynthesis
(S)-3-Hydroxy-3-methylglutaryl-CoA
4.1.3.4
Acetoacetate
4.1.1.4
1.1.1.30
Butanoate metabolism
Acetone
(R)-3-Hydroxybutyrate
00072 2/25/03

FIGURE 6

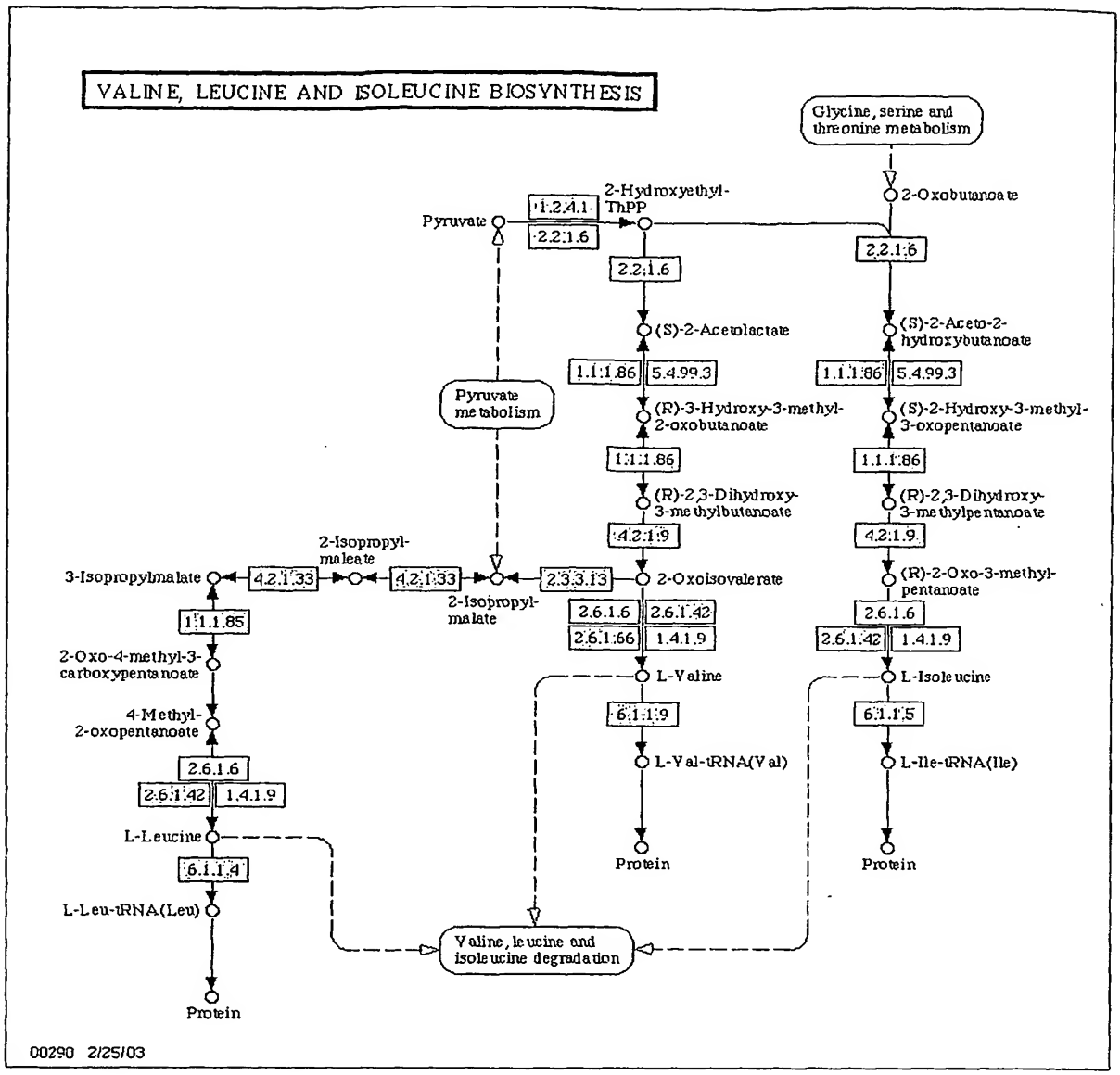
VALINE, LEUCINE AND ISOLEUCINE BIOSYNTHESIS
Glycine, serine and threonine metabolism
2-Oxobutanoate
2-Hydroxyethyl-ThPP
1.2.4.1
Pyruvate
2.2.1.6
2.2.1.6
2.2.1.6
(S)-2-Acetolactate
(S)-2-Aceto-2-hydroxybutanoate
1.1.1.86
5.4.99.3
1.1.1.86
5.4.99.3
Pyruvate metabolism
(R)-3-Hydroxy-3-methyl-2-oxobutanoate
(S)-2-Hydroxy-3-methyl-3-oxopentanoate
1.1.1.86
1.1.1.86
(R)-2,3-Dihydroxy-3-methylbutanoate
(R)-2,3-Dihydroxy-3-methylpentanoate
4.2.1.9
4.2.1.9
2-Isopropylmaleate
3-Isopropylmalate
4.2.1.33
4.2.1.33
2.3.3.13
2-Oxoisovalerate
2-Isopropylmalate
(R)-2-Oxo-3-methylpentanoate
1.1.1.85
2.6.1.6
2.6.1.42
2.6.1.66
1.4.1.9
2.6.1.6
2.6.1.42
1.4.1.9
2-Oxo-4-methyl-3-carboxypentanoate
L-Valine
L-Isoleucine
4-Methyl-2-oxopentanoate
6.1.1.9
6.1.1.5
2.6.1.6
2.6.1.42
1.4.1.9
L-Val-tRNA(Val)
L-Ile-tRNA(Ile)
L-Leucine
Protein
Protein
6.1.1.4
L-Leu-tRNA(Leu)
Valine, leucine and isoleucine degradation
Protein
00290 2/25/03

FIGURE 7

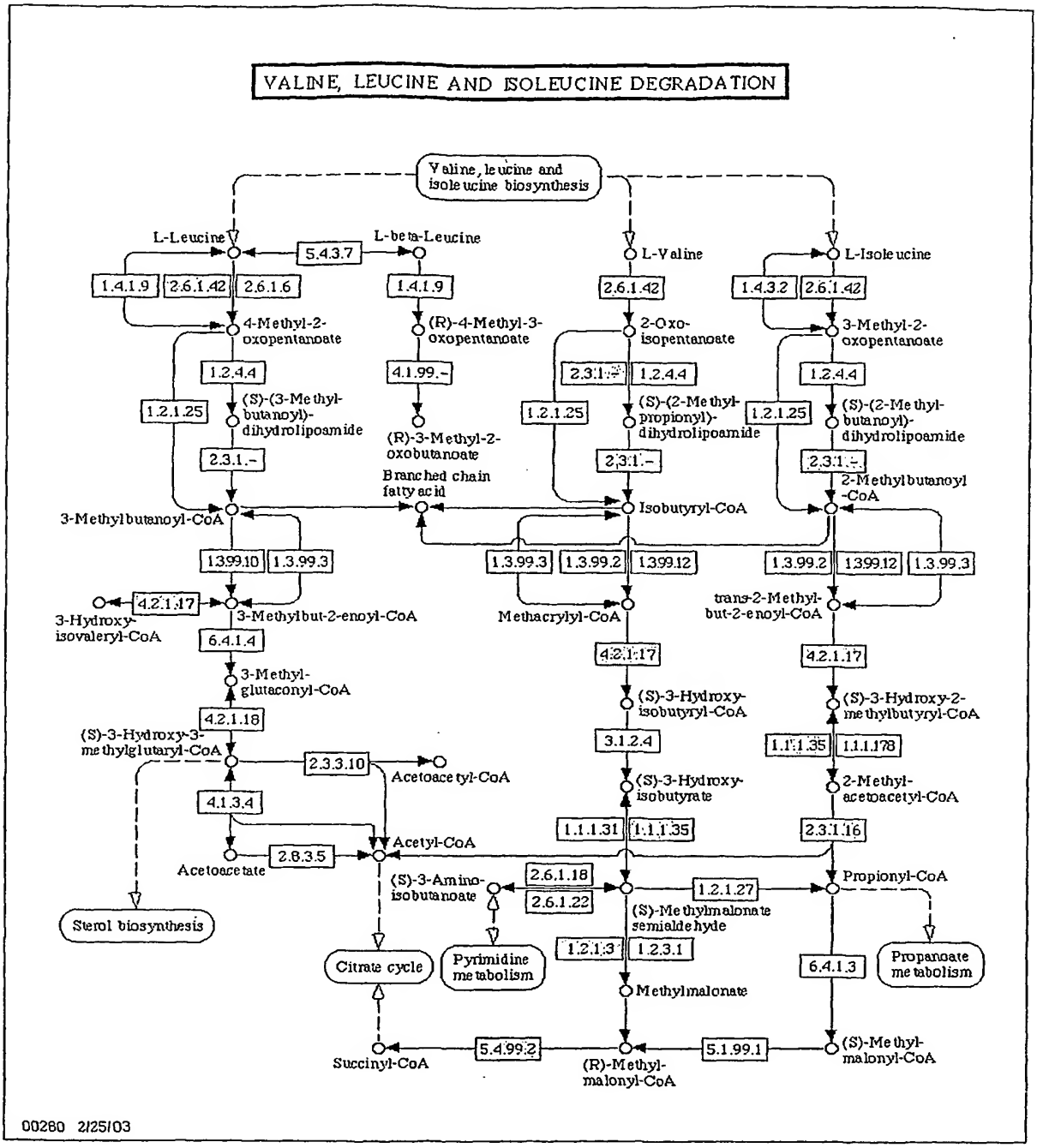
VALINE, LEUCINE AND ISOLEUCINE DEGRADATION
Valine, leucine and isoleucine biosynthesis
L-Leucine
L-beta-Leucine
L-Valine
L-Isoleucine
5.4.3.7
1.4.1.9
2.6.1.42
2.6.1.6
1.4.1.9
2.6.1.42
1.4.3.2
2.6.1.42
4-Methyl-2-oxopentanoate
(R)-4-Methyl-3-oxopentanoate
2-Oxo-isopentanoate
3-Methyl-2-oxopentanoate
1.2.4.4
4.1.99.-
2.3.1.-
1.2.4.4
1.2.4.4
1.2.1.25
(S)-(3-Methyl-butanoyl)-dihydrolipoamide
(R)-3-Methyl-2-oxobutanoate
1.2.1.25
(S)-(2-Methyl-propionyl)-dihydrolipoamide
1.2.1.25
(S)-(2-Methyl-butanoyl)-dihydrolipoamide
2.3.1.-
Branched chain fatty acid
2.3.1.-
2.3.1.-
2-Methylbutanoyl-CoA
3-Methylbutanoyl-CoA
Isobutyryl-CoA
1.3.99.10
1.3.99.3
1.3.99.3
1.3.99.2
1.3.99.12
1.3.99.2
1.3.99.12
1.3.99.3
4.2.1.17
3-Hydroxy-isovaleryl-CoA
3-Methylbut-2-enoyl-CoA
Methacrylyl-CoA
trans-2-Methyl-but-2-enoyl-CoA
6.4.1.4
4.2.1.17
4.2.1.17
3-Methyl-glutaconyl-CoA
(S)-3-Hydroxy-isobutyryl-CoA
(S)-3-Hydroxy-2-methylbutyryl-CoA
4.2.1.18
3.1.2.4
1.1.1.35
1.1.1.178
(S)-3-Hydroxy-3-methylglutaryl-CoA
2.3.3.10
Acetoacetyl-CoA
(S)-3-Hydroxy-isobutyrate
2-Methyl-acetoacetyl-CoA
4.1.3.4
1.1.1.31
1.1.1.35
2.3.1.16
Acetyl-CoA
Acetoacetate
2.8.3.5
2.6.1.18
2.6.1.22
(S)-3-Amino-isobutanoate
1.2.1.27
(S)-Methylmalonate semialdehyde
Propionyl-CoA
Sterol biosynthesis
Citrate cycle
Pyrimidine metabolism
1.2.1.3
1.2.3.1
6.4.1.3
Propanoate metabolism
Methylmalonate
Succinyl-CoA
5.4.99.2
(R)-Methyl-malonyl-CoA
5.1.99.1
(S)-Methyl-malonyl-CoA
00280 2/25/03

FIGURE 8

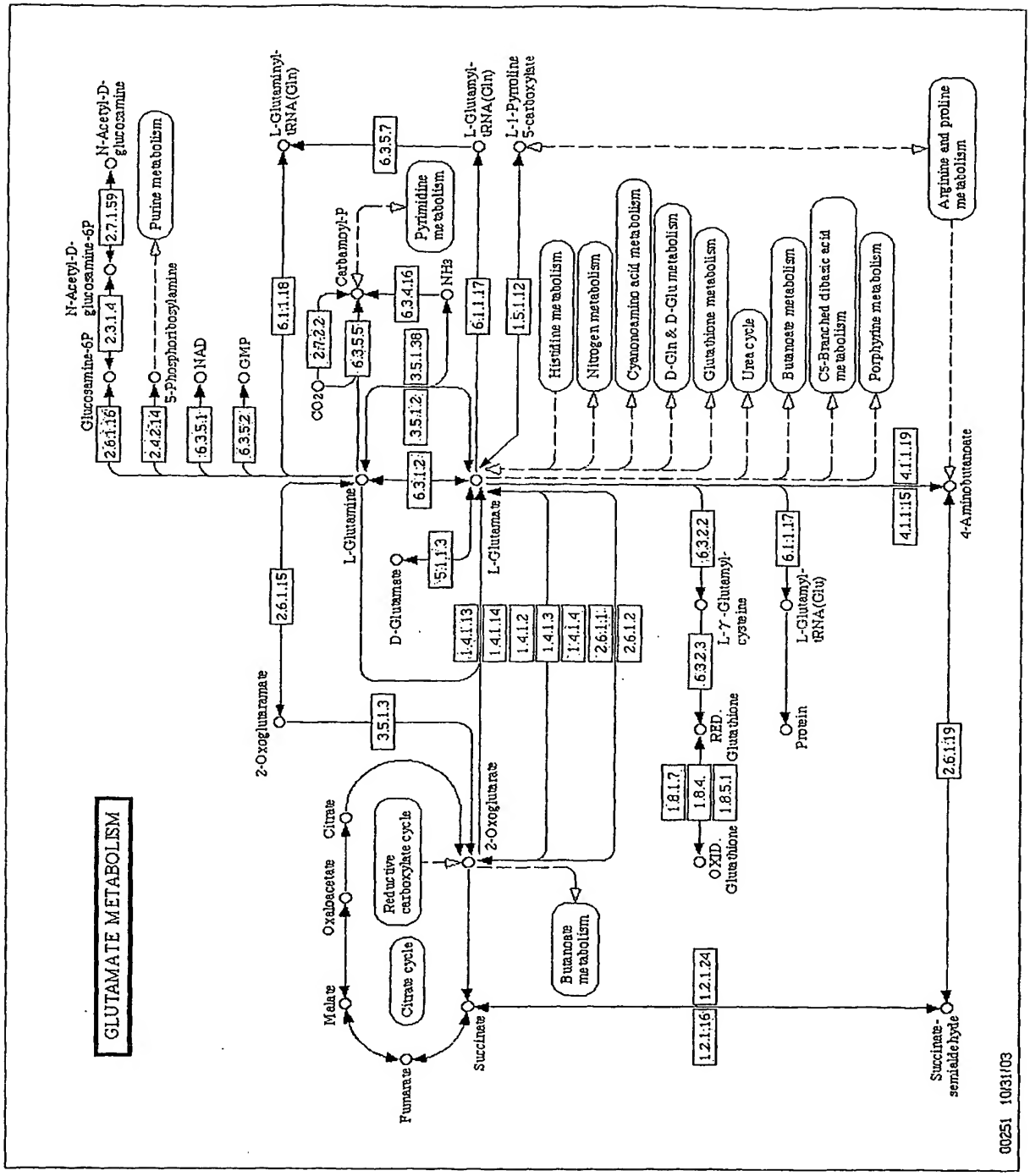
GLUTAMATE METABOLISM
N-Acetyl-D-glucosamine
N-Acetyl-D-glucosamine-6P
Glucosamine-6P
5-Phosphoribosylamine
Purine metabolism
NAD
GMP
CO2
Carbamoyl-P
Pyrimidine metabolism
NH3
L-Glutaminyl-tRNA(Gln)
L-1-Pyrroline 5-carboxylate
Arginine and proline metabolism
Histidine metabolism
Nitrogen metabolism
Cyanoamino acid metabolism
D-Gln & D-Glu metabolism
Glutathione metabolism
Urea cycle
Butanoate metabolism
C5-Branched dibasic acid metabolism
Porphyrine metabolism
L-Glutamine
D-Glutamate
L-Glutamate
4-Aminobutanoate
2-Oxoglutaramate
2-Oxoglutarate
L-γ-Glutamyl-cysteine
L-Glutamyl-tRNA(Glu)
Protein
RED. Glutathione
OXID. Glutathione
Citrate
Oxaloacetate
Malate
Fumarate
Succinate
Reductive carboxylate cycle
Citrate cycle
Butanoate metabolism
Succinate-semialdehyde
2.6.1.16
2.3.1.4
2.7.1.59
2.4.2.14
6.3.5.1
6.3.5.2
6.1.1.18
2.7.2.2
6.3.5.5
6.3.4.16
3.5.1.38
3.5.1.2
6.3.1.2
6.3.5.7
6.1.1.17
1.5.1.12
5.1.1.3
2.6.1.15
3.5.1.3
1.4.1.13
1.4.1.14
1.4.1.2
1.4.1.3
1.4.1.4
2.6.1.1
2.6.1.2
6.3.2.2
6.3.2.3
1.8.1.7
1.8.4
1.8.5.1
6.1.1.17
4.1.1.15
4.1.1.19
2.6.1.19
1.2.1.16
1.2.1.24
00251 10/31/03

FIGURE 9

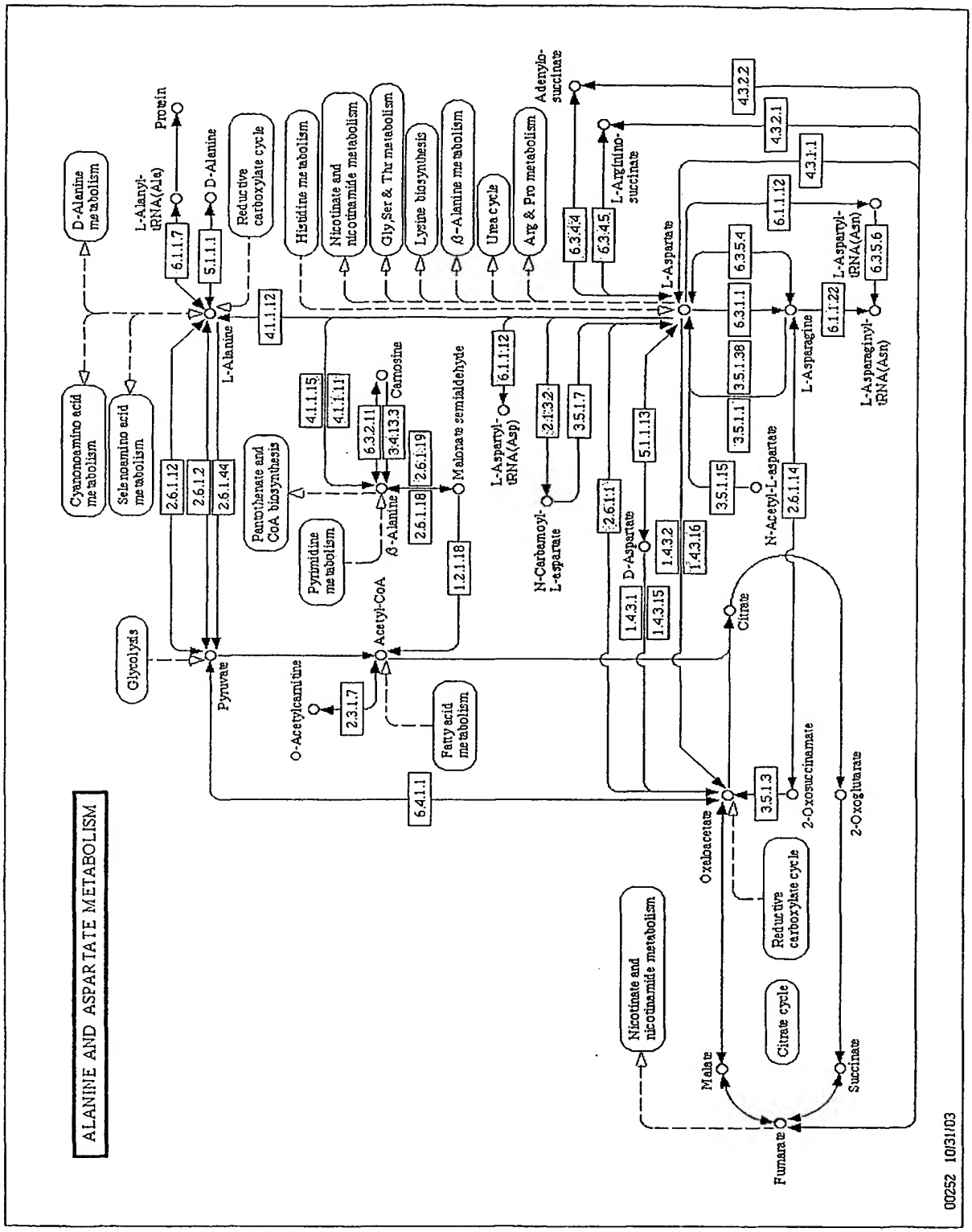
ALANINE AND ASPARTATE METABOLISM
Protein
D-Alanine metabolism
L-Alanyl-tRNA(Ala)
D-Alanine
Reductive carboxylate cycle
Histidine metabolism
Nicotinate and nicotinamide metabolism
Gly,Ser & Thr metabolism
Lysine biosynthesis
β-Alanine metabolism
Urea cycle
Arg & Pro metabolism
Adenylo-succinate
L-Argininosuccinate
L-Aspartate
L-Asparagine
L-Asparagine-tRNA(Asn)
L-Asparagine-tRNA(Asn)
Cyanoamino acid metabolism
Selenoamino acid metabolism
L-Alanine
Pantothenate and CoA biosynthesis
Pyrimidine metabolism
Carnosine
β-Alanine
Malonate semialdehyde
L-Asparagine-tRNA(Asp)
N-Carbamoyl-L-asparate
D-Aspartate
N-Acetyl-L-aspartate
Glycolysis
Pyruvate
Acetyl-CoA
O-Acetylcarnitine
Fatty acid metabolism
Citrate
Oxaloacetate
2-Oxosuccinamate
2-Oxoglutarate
Nicotinate and nicotinamide metabolism
Reductive carboxylate cycle
Citrate cycle
Malate
Fumarate
Succinate
00252 10/31/03

FIGURE 10

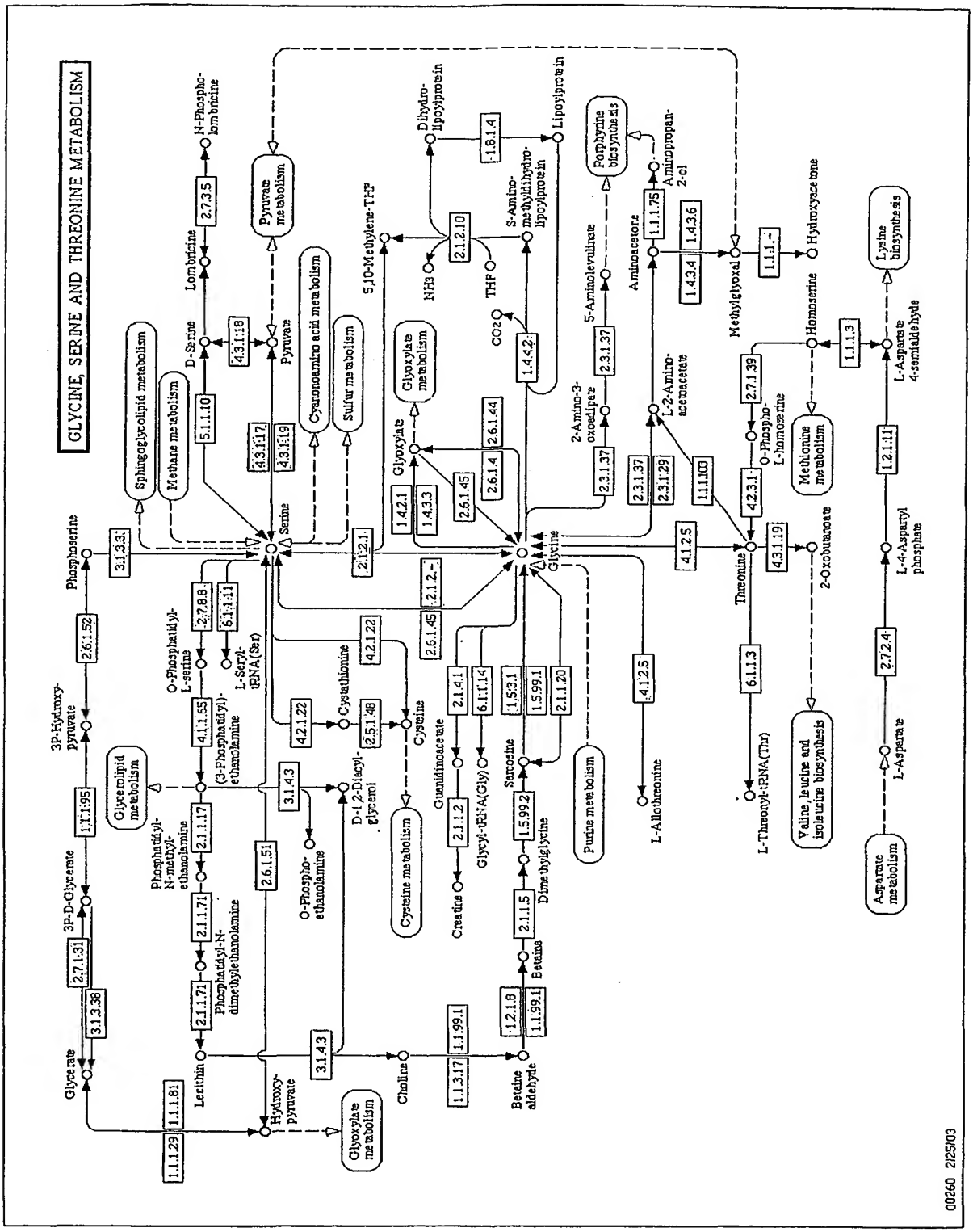
GLYCINE, SERINE AND THREONINE METABOLISM
Glycine
Serine
Threonine
Glyoxylate
Pyruvate
Glycerate
Choline
Betaine
Betaine aldehyde
Creatine
Sarcosine
Dimethylglycine
Cysteine
Homoserine
Methylglyoxal
Hydroxyacetone
Aminoacetone
Aminopropan-2-ol
Lipoylprotein
Dihydro-lipoylprotein
S-Aminomethyldihydro-lipoylprotein
5,10-Methylene-THF
THF
NH3
CO2
Lombricine
N-Phospho-lombricine
D-Serine
Phosphoserine
Phosphohydroxy-pyruvate
3P-Hydroxy-pyruvate
3P-D-Glycerate
O-Phosphatidyl-L-serine
L-Seryl-tRNA(Ser)
(3-Phosphatidyl)-ethanolamine
Phosphatidyl-N-methyl-ethanolamine
Phosphatidyl-N-dimethylethanolamine
O-Phospho-ethanolamine
D-1,2-Diacyl-glycerol
Cystathionine
Guanidinoacetate
Glycyl-tRNA(Gly)
L-Threonyl-tRNA(Thr)
L-Allothreonine
2-Amino-3-oxoadipate
5-Aminolevulinate
L-2-Amino-acetoacetate
O-Phospho-L-homoserine
2-Oxobutanoate
L-4-Aspartyl phosphate
L-Aspartate
Glycerolipid metabolism
Sphingoglycolipid metabolism
Methane metabolism
Pyruvate metabolism
Cyanoamino acid metabolism
Sulfur metabolism
Glyoxylate metabolism
Cysteine metabolism
Purine metabolism
Porphyrine biosynthesis
Methionine metabolism
Valine, leucine and isoleucine biosynthesis
Lysine biosynthesis
Aspartate metabolism
00260 2125/03

FIGURE 11

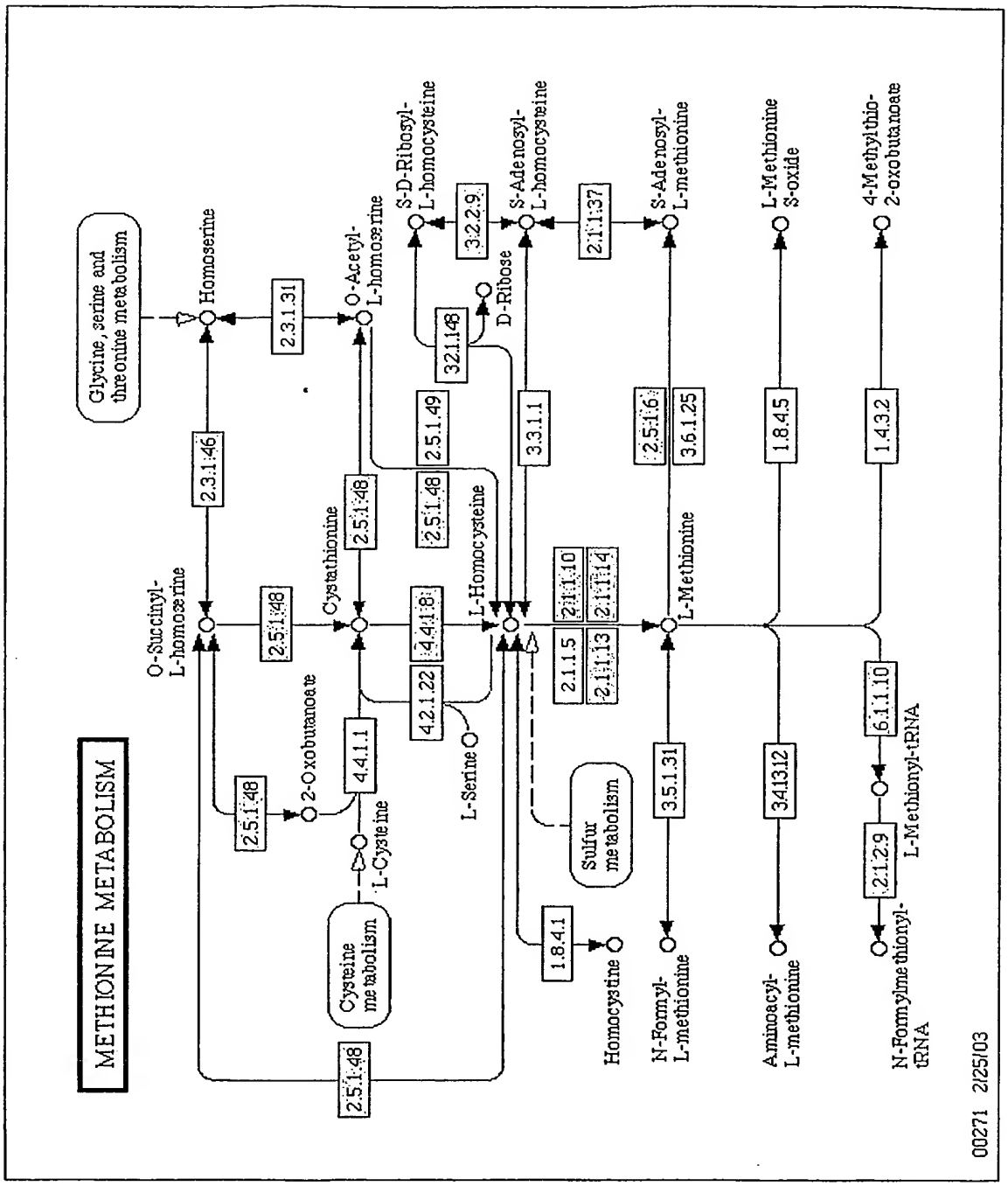
METHIONINE METABOLISM
Glycine, serine and threonine metabolism
Homoserine
O-Succinyl-L-homoserine
O-Acetyl-L-homoserine
Cystathionine
L-Homocysteine
L-Cysteine
2-Oxobutanoate
L-Serine
D-Ribose
S-D-Ribosyl-L-homocysteine
S-Adenosyl-L-homocysteine
S-Adenosyl-L-methionine
L-Methionine
L-Methionine S-oxide
4-Methylthio-2-oxobutanoate
Homocysteine
N-Formyl-L-methionine
Aminoacyl-L-methionine
N-Formylmethionyl-tRNA
L-Methionyl-tRNA
Cysteine metabolism
Sulfur metabolism
2.3.1.46
2.3.1.31
2.5.1.48
2.5.1.49
3.2.2.9
3.2.1.48
3.3.1.1
2.1.1.37
2.5.1.6
3.6.1.25
1.8.4.5
1.4.3.2
6.1.1.10
2.1.2.9
3.5.1.31
3.4.13.12
4.4.1.8
4.2.1.22
4.4.1.1
2.1.1.5
2.1.1.13
2.1.1.10
2.1.1.14
1.8.4.1
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FIGURE 12

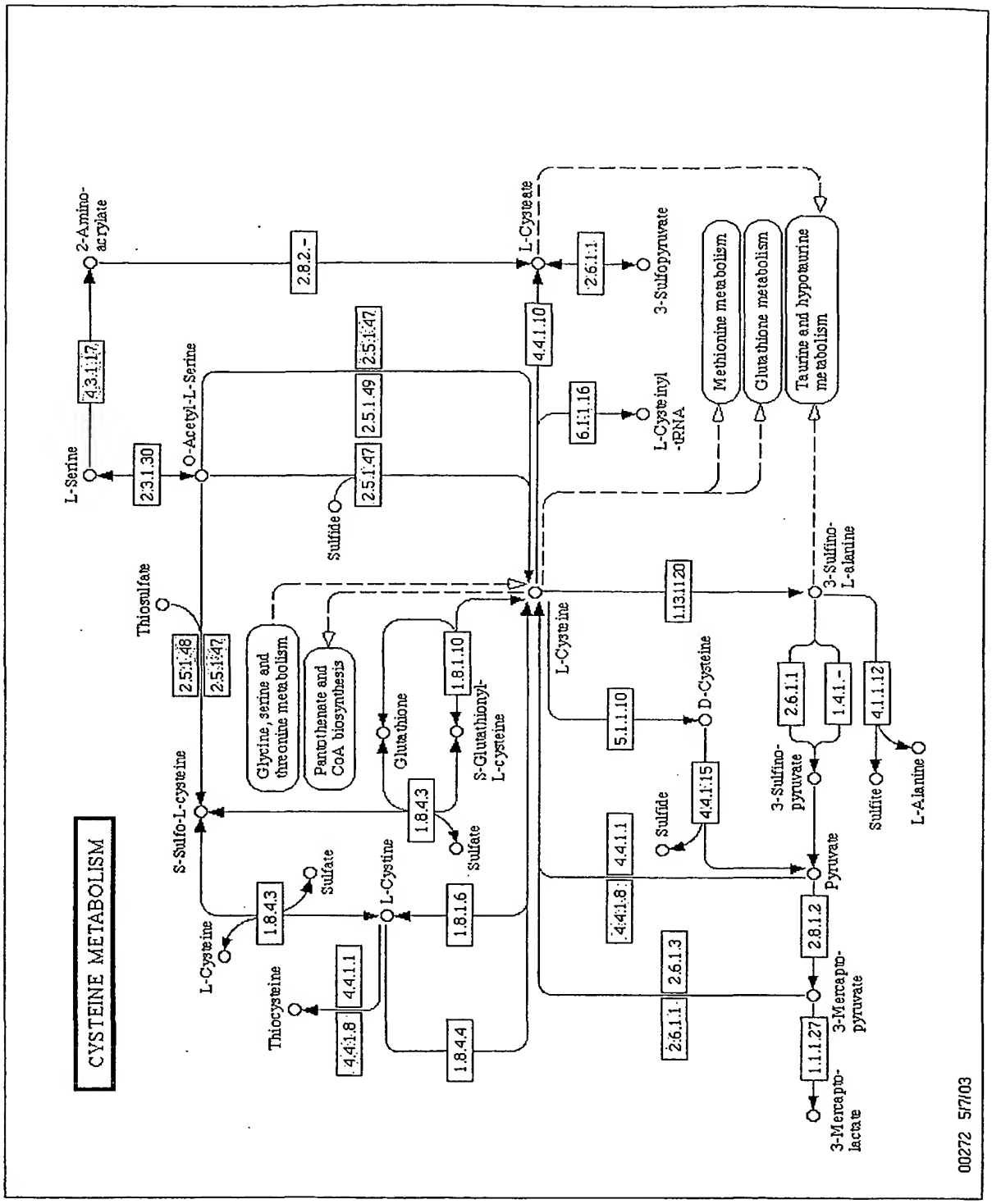

CYSTEINE METABOLISM
2-Amino-acrylate
4.3.1.17
L-Serine
2.3.1.30
O-Acetyl-L-Serine
2.8.2.-
L-Cysteate
2.6.1.1
3-Sulfopyruvate
4.4.1.10
2.5.1.47
2.5.1.49
2.5.1.47
6.1.1.16
L-Cysteinyl-tRNA
Methionine metabolism
Glutathione metabolism
Taurine and hypotaurine metabolism
Sulfide
Thiosulfate
2.5.1.48
2.5.1.47
Glycine, serine and threonine metabolism
Pantothenate and CoA biosynthesis
Glutathione
1.8.1.10
S-Glutathionyl-L-cysteine
L-Cysteine
1.13.11.20
3-Sulfino-L-alanine
S-Sulfo-L-cysteine
1.8.4.3
Sulfate
5.1.1.10
D-Cysteine
2.6.1.1
1.4.1.-
4.1.1.12
L-Alanine
L-Cystine
1.8.4.3
Sulfate
1.8.1.6
4.4.1.15
Sulfide
3-Sulfino-pyruvate
Sulfite
L-Cysteine
4.4.1.1
4.4.1.8
Thiocysteine
4.4.1.1
4.4.1.8
1.8.4.4
2.6.1.3
2.6.1.1
2.8.1.2
Pyruvate
3-Mercapto-pyruvate
1.1.1.27
3-Mercapto-lactate
00272 5/7/03

FIGURE 13

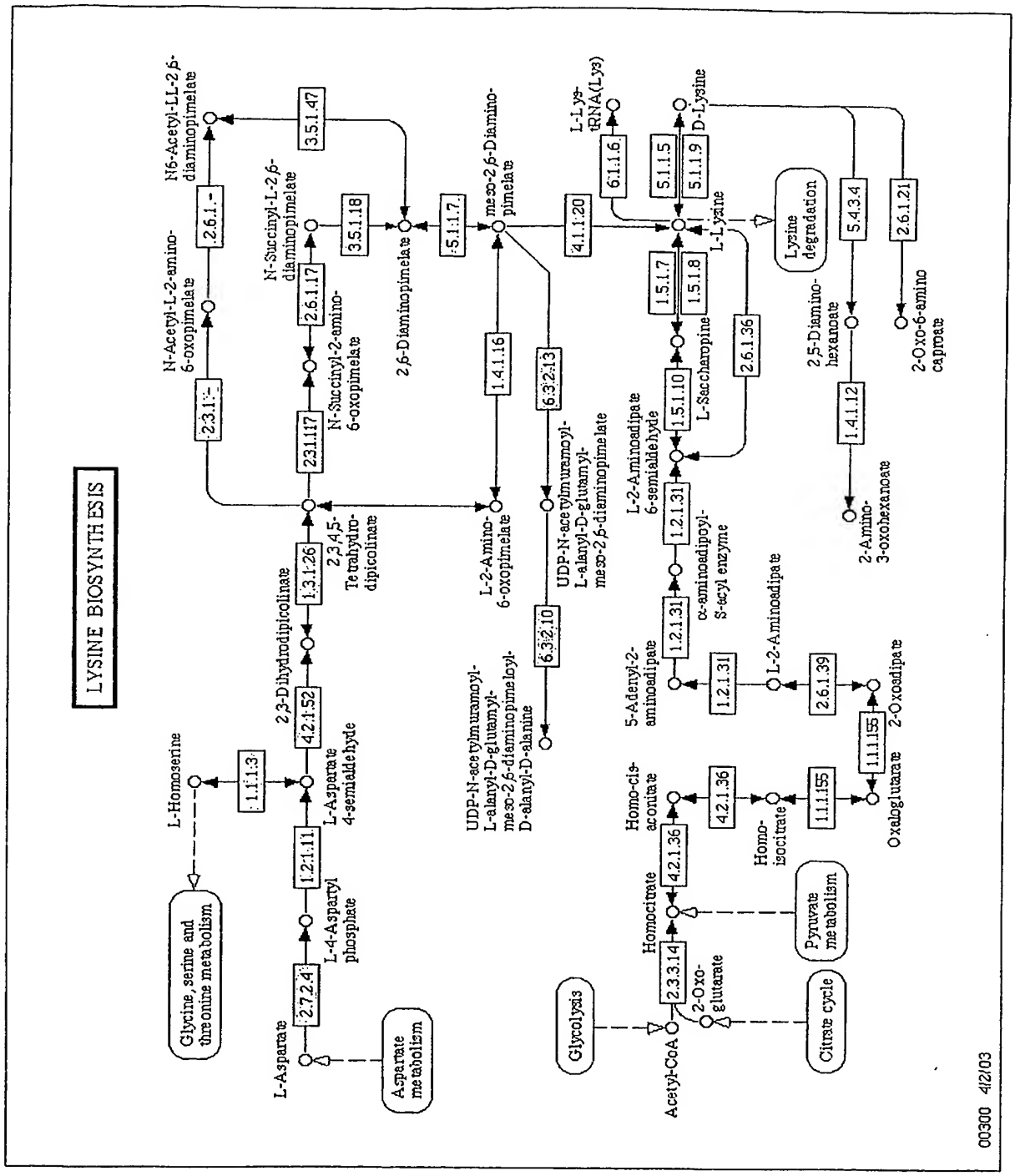

LYSINE BIOSYNTHESIS
L-Homoserine
Glycine, serine and threonine metabolism
1.1.1.3
L-Aspartate
2.7.2.4
L-4-Aspartyl phosphate
1.2.1.11
L-Aspartate 4-semialdehyde
Aspartate metabolism
2,3-Dihydrodipicolinate
4.2.1.52
2,3,4,5-Tetrahydro-dipicolinate
1.3.1.26
N-Acetyl-L-2-amino-6-oxopimelate
2.3.1.-
N6-Acetyl-LL-2,6-diaminopimelate
2.6.1.-
N-Succinyl-L-2,6-diaminopimelate
3.5.1.47
N-Succinyl-2-amino-6-oxopimelate
2.6.1.17
2,6-Diaminopimelate
3.5.1.18
5.1.1.7
meso-2,6-Diamino-pimelate
L-2-Amino-6-oxopimelate
1.4.1.16
UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-alanine
6.3.2.10
UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimelate
6.3.2.13
4.1.1.20
L-Lys-tRNA(Lys)
6.1.1.6
5.1.1.5
5.1.1.9
D-Lysine
L-Lysine
1.5.1.7
1.5.1.8
1.5.1.10
L-Saccharopine
2.6.1.36
L-2-Aminoadipate 6-semialdehyde
1.2.1.31
α-aminoadipoyl-S-acyl enzyme
1.2.1.31
5-Adenyl-2-aminoadipate
1.2.1.31
L-2-Aminoadipate
2.6.1.39
Lysine degradation
2,5-Diamino-hexanoate
5.4.3.4
1.4.1.12
2-Amino-3-oxohexanoate
2-Oxo-6-amino caproate
2.6.1.21
Glycolysis
Acetyl-CoA
2.3.3.14
Homocitrate
4.2.1.36
Homo-cis-aconitate
4.2.1.36
Homo-isocitrate
1.1.1.155
Oxalglutarate
1.1.1.155
2-Oxoadipate
2-Oxo-glutarate
Citrate cycle
Pyruvate metabolism
00300 4/2/03

FIGURE 14

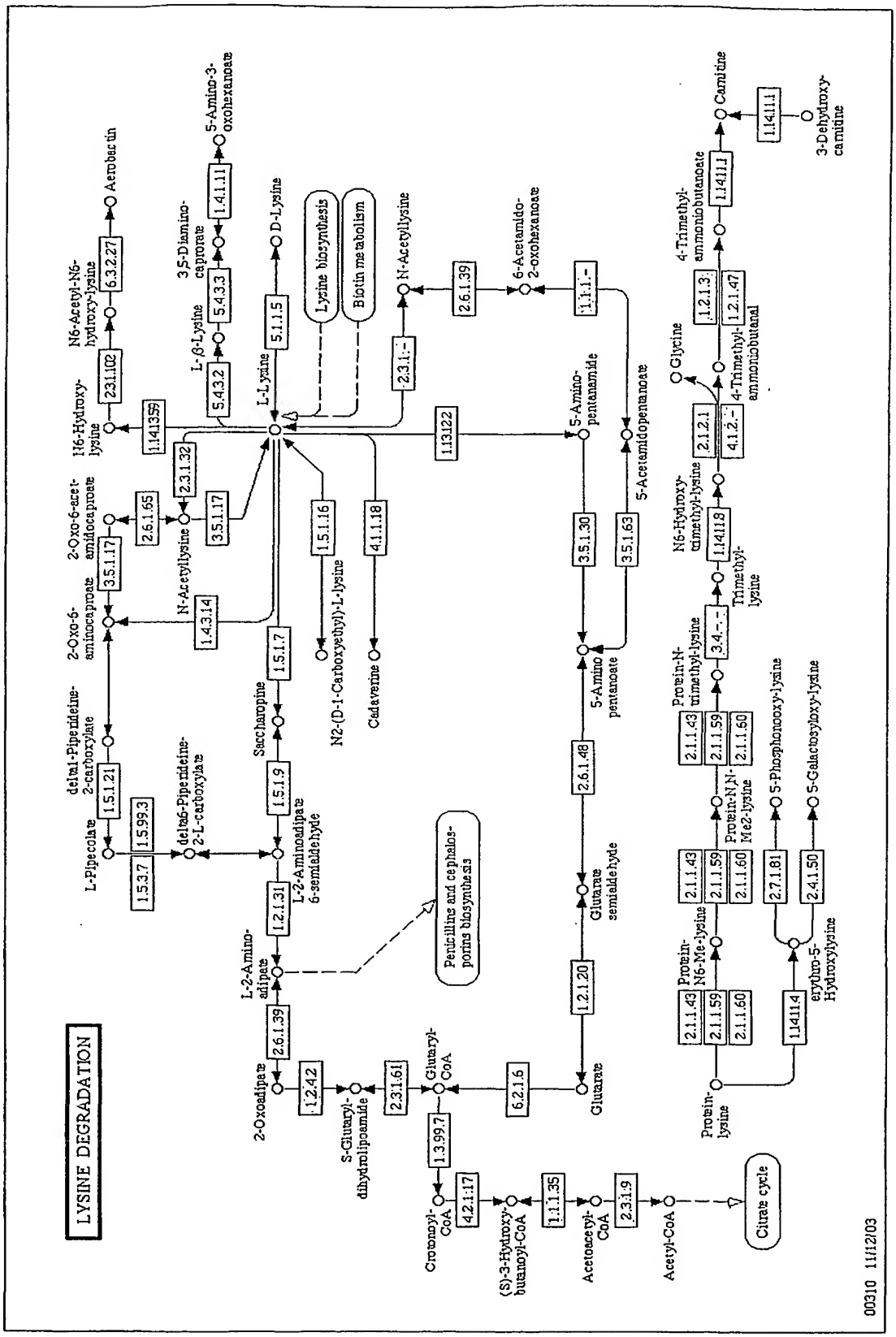

LYSINE DEGRADATION
L-Pipecolate
delta1-Piperideine-2-carboxylate
delta6-Piperideine-2-L-carboxylate
2-Oxo-6-amino caproate
L-2-Aminoadipate 6-semialdehyde
Saccharopine
N2-(D-1-Carboxyethyl)-L-lysine
Cadaverine
N-Acetyllysine
2-Oxo-6-acet-amidocaproate
N6-Hydroxy-lysine
N6-Acetyl-N6-hydroxy-lysine
Aerobactin
L-β-Lysine
3,5-Diamino-caproate
5-Amino-3-oxohexanoate
L-Lysine
D-Lysine
Lysine biosynthesis
Biotin metabolism
N-Acetyllysine
6-Acetamido-2-oxohexanoate
5-Amino-pentanamide
5-Acetamidopentanoate
5-Amino pentanoate
Glycine
4-Trimethyl-ammoniobutanal
4-Trimethyl-ammoniobutanoate
Carnitine
3-Dehydroxy-carnitine
N6-Hydroxy-trimethyl-lysine
Trimethyl-lysine
Protein-N-trimethyl-lysine
Protein-NN-Me2-lysine
Protein-N6-Me-lysine
Protein-lysine
5-Phosphonooxy-lysine
5-Galactosyloxy-lysine
erythro-5-Hydroxylysine
Penicillins and cephalosporins biosynthesis
Glutaryl-CoA
S-Glutaryl-dihydrolipoamide
2-Oxoadipate
L-2-Amino-adipate
Glutarate semialdehyde
Glutarate
Crotonoyl-CoA
(S)-3-Hydroxy-butanoyl-CoA
Acetoacetyl-CoA
Acetyl-CoA
Citrate cycle
00310 11/12/03

FIGURE 15

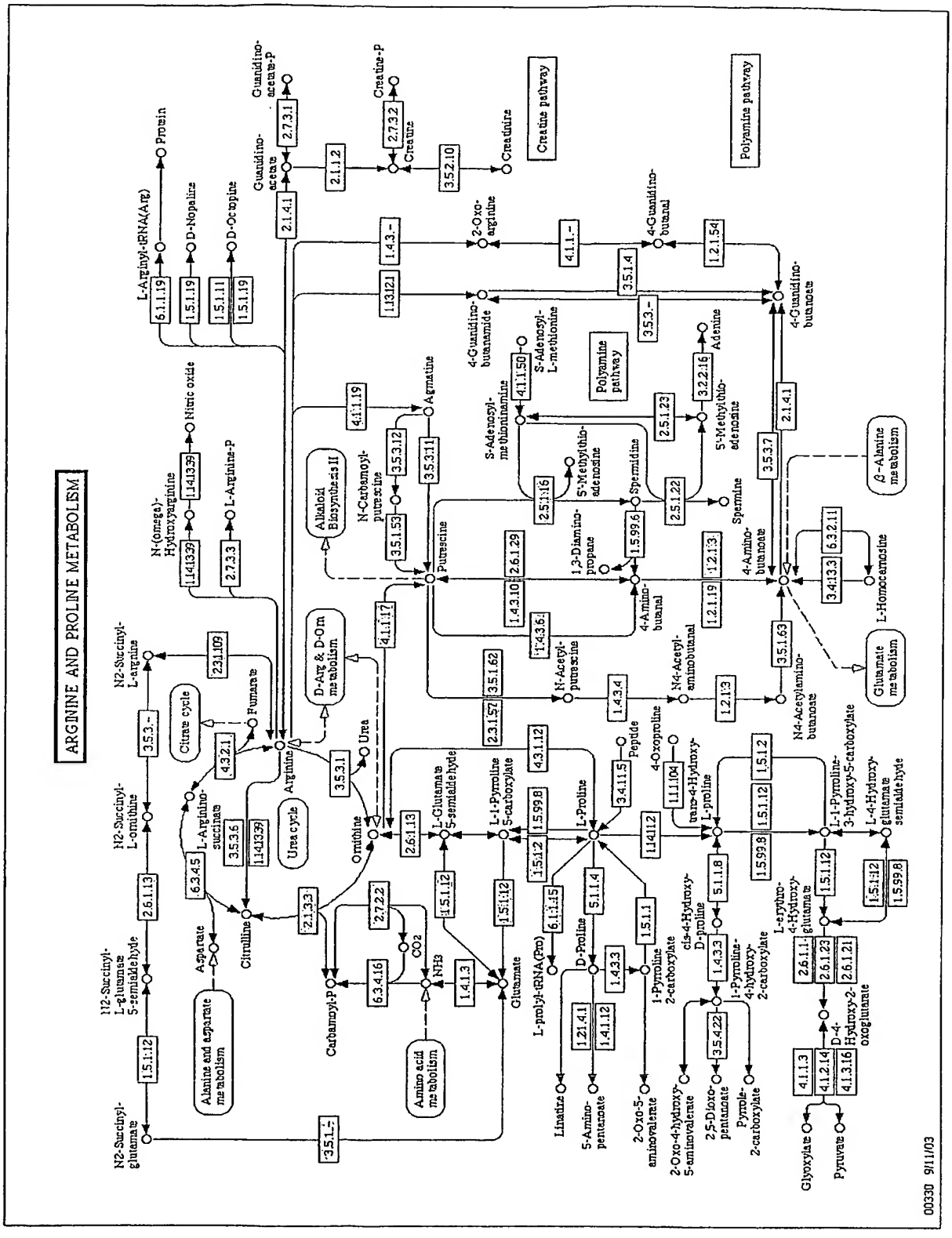

ARGINE AND PROLINE METABOLISM
Protein
L-Arginyl-tRNA(Arg)
D-Nopaline
D-Octopine
Guanidino-acetate
Guanidino-acetate-P
Creatine-P
Creatine
Creatinine
Creatine pathway
Polyamine pathway
Nitric oxide
N(omega)-Hydroxyarginine
L-Arginine-P
Agmatine
Alkaloid Biosynthesis II
N-Carbamoyl-putrescine
Putrescine
2-Oxo-arginine
4-Guanidino-butanamide
4-Guanidino-butanal
4-Guanidino-butanoate
S-Adenosyl-methioninamine
S-Adenosyl-L-methionine
5'-Methylthio-adenosine
Adenine
Spermidine
Spermine
1,3-Diamino-propane
4-Amino-butanal
4-Amino-butanoate
β-Alanine metabolism
L-Homocarnosine
Glutamate metabolism
N-Acetyl-putrescine
N4-Acetyl-aminobutanal
N4-Acetylamino-butanoate
Peptide
4-Oxoproline
trans-4-Hydroxy-L-proline
L-Proline
L-1-Pyrroline 5-carboxylate
L-Glutamate 5-semialdehyde
Ornithine
Urea
Arginine
Fumarate
Citrulline
Aspartate
L-Arginino-succinate
Citrate cycle
Urea cycle
D-Arg & D-Orn metabolism
N2-Succinyl-L-arginine
N2-Succinyl-L-ornithine
N2-Succinyl-L-glutamate 5-semialdehyde
N2-Succinyl-glutamate
Alanine and aspartate metabolism
Amino acid metabolism
Carbamoyl-P
Glutamate
L-prolyl-tRNA(Pro)
D-Proline
Linaltre
5-Amino-pentenoate
1-Pyrroline 2-carboxylate
2-Oxo-5-aminovalerate
2-Oxo-4-hydroxy-5-aminovalerate
2,5-Dioxo-pentanoate
cis-4-Hydroxy-D-proline
1-Pyrroline-4-hydroxy-2-carboxylate
Pyrrole-2-carboxylate
L-erythro-4-Hydroxy-glutamate
L-1-Pyrroline-3-hydroxy-5-carboxylate
L-4-Hydroxy-glutamate semialdehyde
D-4-Hydroxy-2-oxoglutarate
Glyoxylate
Pyruvate
00330 9/11/03

FIGURE 16

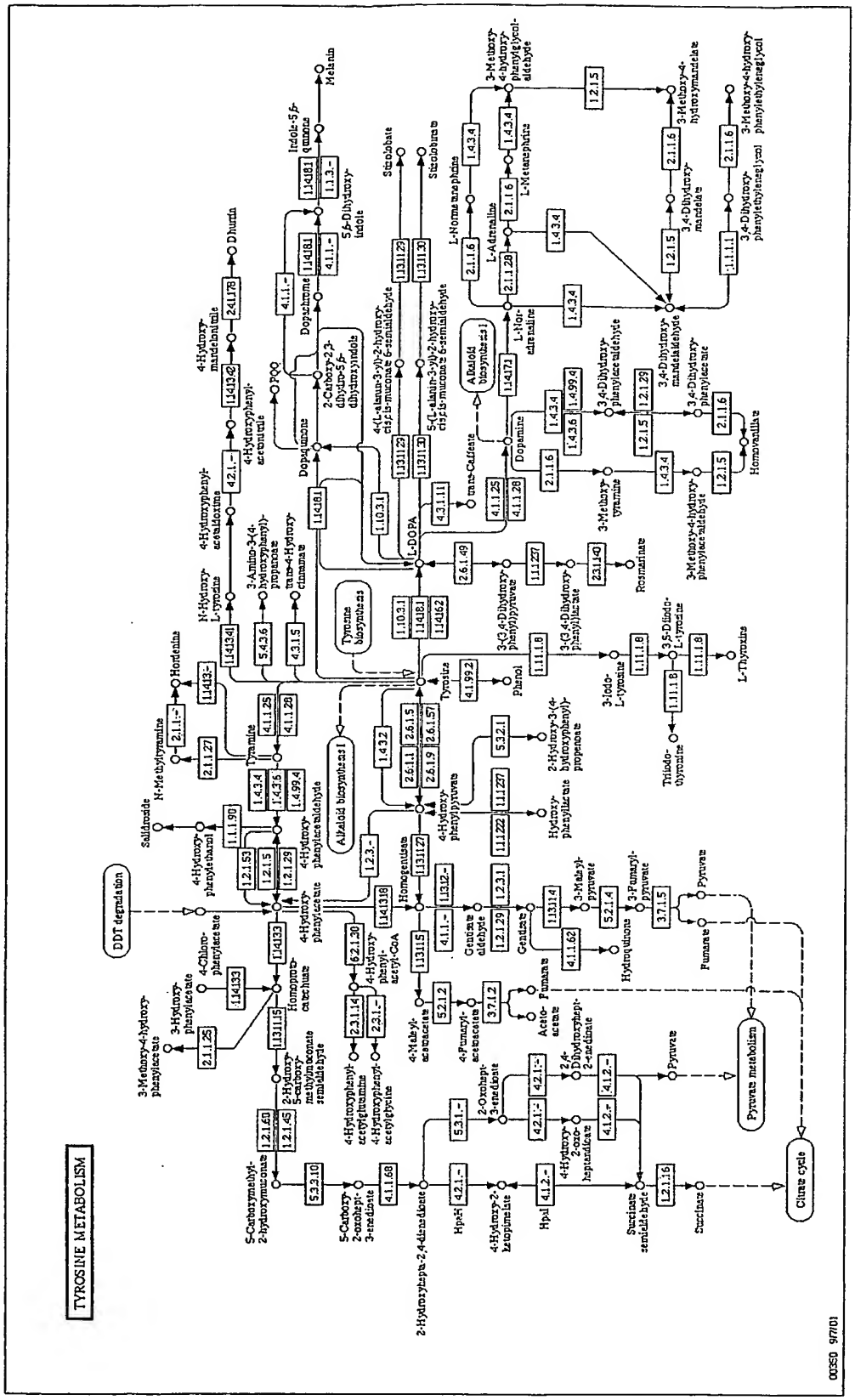

TYROSINE METABOLISM
DDT degradation
Citrate cycle
Pyruvate metabolism
Tyrosine
L-DOPA
Dopamine
Dopaquinone
Melanin
Indole-5,6-quinone
5,6-Dihydroxyindole
Dopachrome
Norepinephrine
Epinephrine
L-Normetanephrine
L-Metanephrine
L-Adrenaline
L-Noradrenaline
Homovanillate
3-Methoxy-4-hydroxyphenylglycol
3,4-Dihydroxyphenylglycol
3-Methoxy-4-hydroxymandelate
3,4-Dihydroxymandelate
3-Methoxy-4-hydroxyphenylacetaldehyde
3,4-Dihydroxyphenylacetaldehyde
3-Methoxy-4-hydroxyphenylacetate
3,4-Dihydroxyphenylacetate
3-Methoxy-4-hydroxymandelaldehyde
3,4-Dihydroxymandelaldehyde
3-Methoxy-4-hydroxyphenylglycolaldehyde
3-Methoxy-4-hydroxyphenylethyleneglycol
3,4-Dihydroxyphenylethyleneglycol
3-Methoxytyramine
3,4-Dihydroxyphenylacetaldehyde
Rosmarinate
3-(3,4-Dihydroxyphenyl)pyruvate
3-(3,4-Dihydroxyphenyl)lactate
3-Iodo-L-tyrosine
3,5-Diiodo-L-tyrosine
L-Thyroxine
Triiodothyronine
Tyramine
N-Hydroxy-L-tyrosine
4-Hydroxyphenylacetaldoxime
4-Hydroxymandelonitrile
Dhurrin
4-Hydroxyphenylacetonitrile
3-Amino-3-(4-hydroxyphenyl)propanoate
trans-4-Hydroxycinnamate
Tyrosine biosynthesis
Alkaloid biosynthesis I
Alkaloid biosynthesis II
Stizolobate
Stizolobinate
4-(L-alanin-3-yl)-2-hydroxy-cis,cis-muconate 6-semialdehyde
5-(L-alanin-3-yl)-2-hydroxy-cis,cis-muconate 6-semialdehyde
trans-Caffeate
2-Carboxy-2,3-dihydro-5,6-dihydroxyindole
POO
Hordenine
N-Methyltyramine
Salsolinoside
4-Hydroxyphenylethanol
4-Hydroxyphenylacetate
4-Hydroxyphenylacetaldehyde
4-Hydroxyphenylpyruvate
Homogentisate
Genisate aldehyde
Genistate
3-Methylpyruvate
3-Fumarylpyruvate
Hydroxyphenylpyruvate
2-Hydroxy-3-(4-hydroxyphenyl)propenoate
Phenol
Fumarate
Pyruvate
Acetoacetate
Homogentisate 1,2-dioxygenase
Homogentisate 1,2-dioxygenase (decarboxylating)
4-Maleylacetoacetate
4-Fumarylacetoacetate
4-Hydroxyphenylacetyl-CoA
4-Hydroxyphenylacetylglutamine
4-Hydroxyphenylacetylglycine
Homoprotocatechuate
2-Hydroxy-5-carboxymethylmuconate semialdehyde
4-Chlorophenylacetate
3-Hydroxyphenylacetate
3-Methoxy-4-hydroxyphenylacetate
5-Carboxymethyl-2-hydroxymuconate
5-Carboxy-2-oxohept-3-enedioate
4-Hydroxyphenylacetylglutamine
4-Hydroxyphenylacetylglycine
2-Hydroxybuta-2,4-dienedioate
2-Oxohept-3-enedioate
4-Hydroxy-2-oxoheptanedioate
2,6-Dioxohept-4-enedioate
Succinate semialdehyde
Succinate
HpaH
HpaI
00350 9/7/01

FIGURE 17

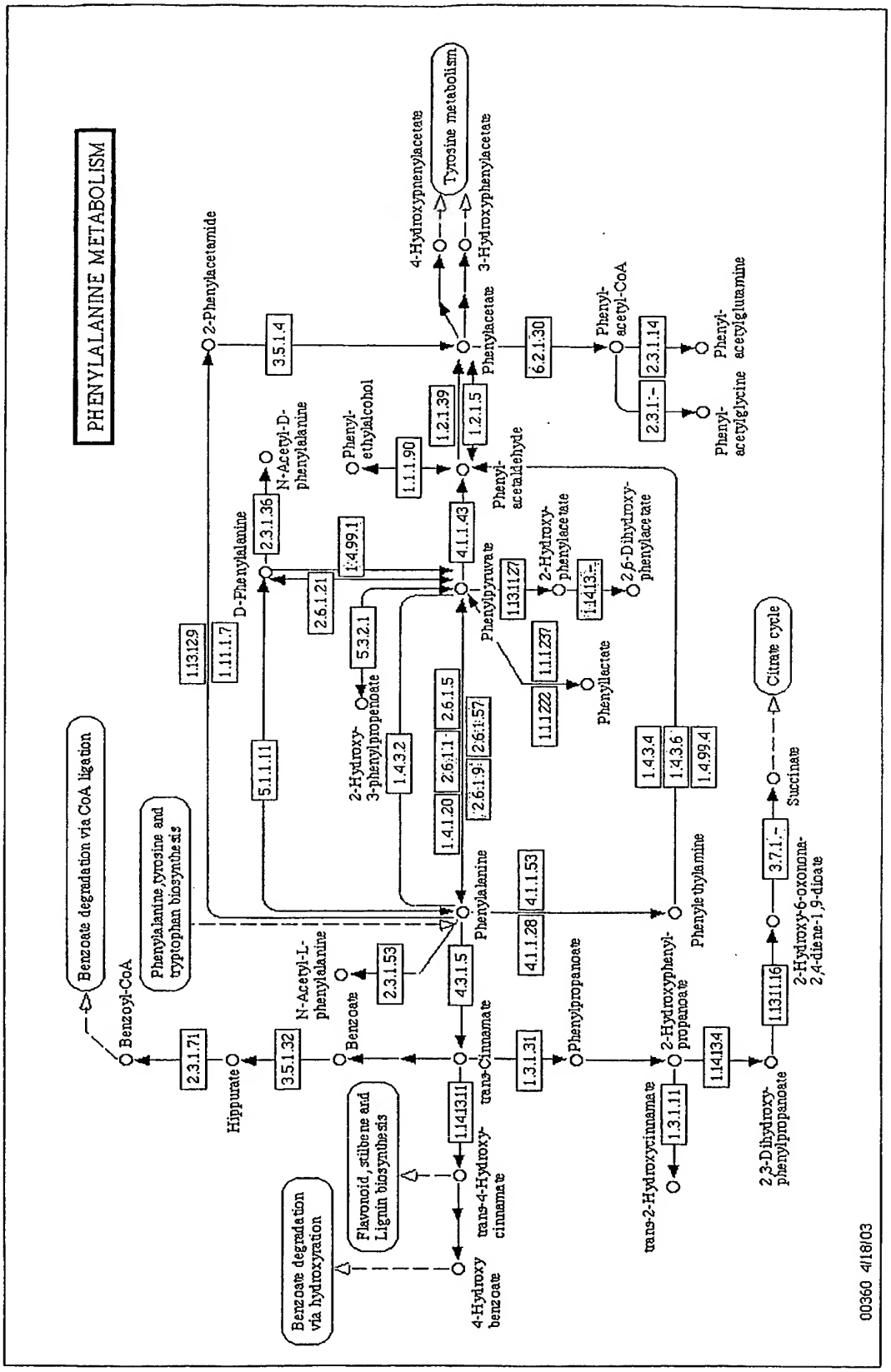

PHENYLALANINE METABOLISM
Benzoate degradation via CoA ligation
Benzoyl-CoA
Phenylalanine,tyrosine and tryptophan biosynthesis
Hippurate
N-Acetyl-L-phenylalanine
Benzoate
Cinnamate
trans-Cinnamate
Phenylpropanoate
Phenylalanine
Phenylethylamine
2-Hydroxyphenyl-propanoate
trans-2-Hydroxycinnamate
2,3-Dihydroxy-phenylpropanoate
2-Hydroxy-6-oxonona-2,4-diene-1,9-dicate
Succinate
Citrate cycle
Benzoate degradation via hydroxylation
Flavonoid, stilbene and Lignin biosynthesis
4-Hydroxy benzoate
trans-4-Hydroxy-cinnamate
2-Phenylacetamide
D-Phenylalanine
N-Acetyl-D-phenylalanine
Phenyl-ethylalcohol
Phenyl-acetaldehyde
Phenylacetate
4-Hydroxyphenylacetate
3-Hydroxyphenylacetate
Tyrosine metabolism
Phenyl-acetyl-CoA
Phenyl-acetylglycine
Phenyl-acetylglutamine
Phenylpyruvate
2-Hydroxy-phenylacetate
2,6-Dihydroxy-phenylacetate
Phenyllactate
2-Hydroxy-3-phenylpropenoate
3.5.1.4
6.2.1.30
2.3.1.14
2.3.1.-
1.2.1.39
1.2.1.5
1.1.1.90
2.3.1.36
1.4.99.1
4.1.1.43
2.6.1.21
5.3.2.1
1.13.11.27
1.14.13.-
1.1.1.237
1.1.1.222
1.13.12.9
1.11.1.7
5.1.1.11
1.4.3.2
2.6.1.11
2.6.1.5
2.6.1.9
2.6.1.57
1.4.1.20
2.3.1.53
4.3.1.5
4.1.1.28
4.1.1.53
1.3.1.31
1.14.13.11
1.3.1.11
1.14.13.4
1.14.13.6
1.14.99.4
1.14.13.4
1.13.11.16
3.7.1.-
00360 4/18/03

FIGURE 18

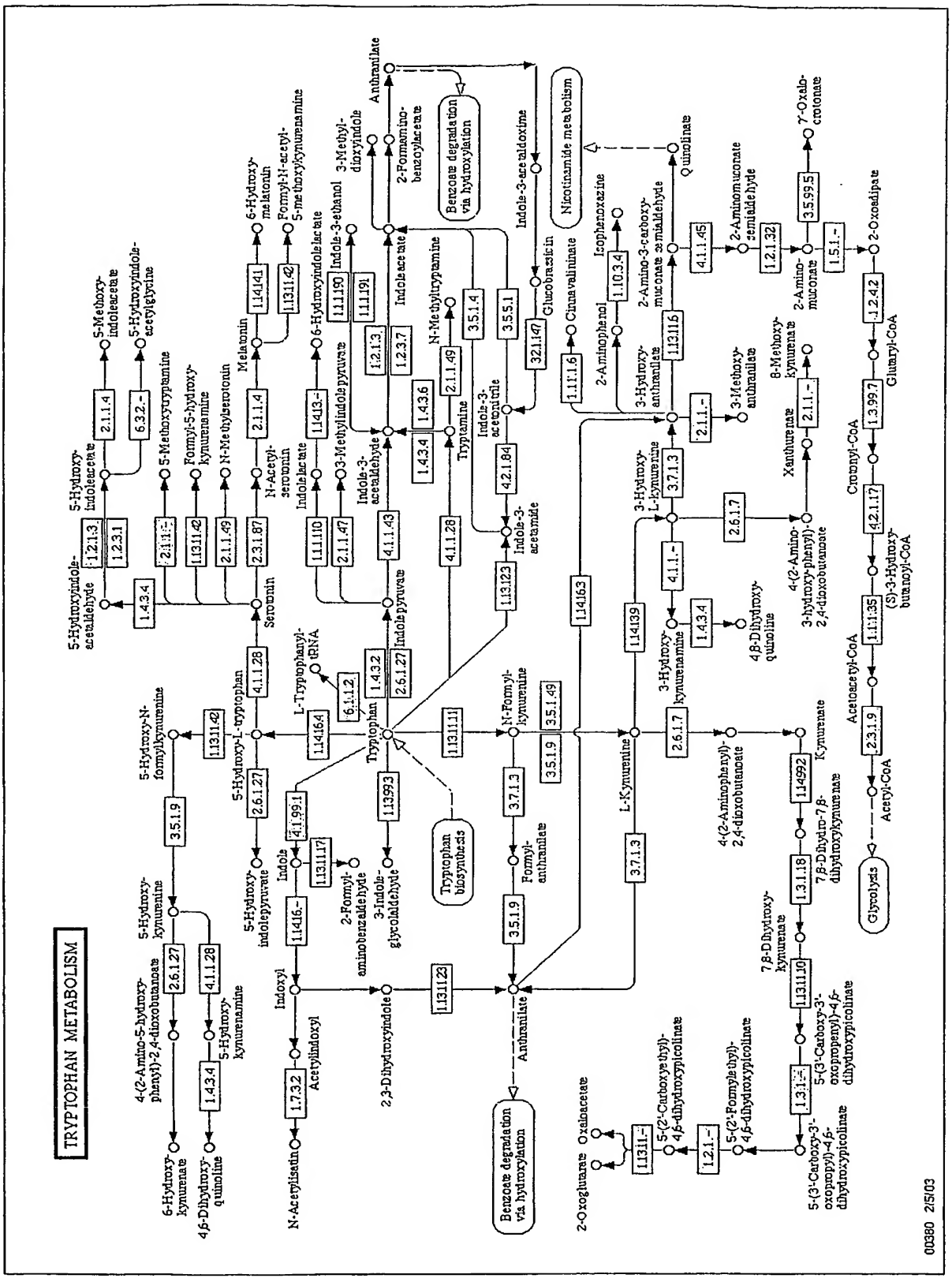

TRYPTOPHAN METABOLISM
Tryptophan
L-Tryptophanyl-tRNA
Indolepyruvate
Indole-3-acetaldehyde
Indole-3-acetamide
Indole-3-acetonitrile
Indole-3-acetaldoxime
Indole-3-acetate
Tryptamine
N-Methyltryptamine
Serotonin
N-Acetylserotonin
Melatonin
6-Hydroxymelatonin
Formyl-N-acetyl-5-methoxykynuramine
5-Hydroxyindole-acetaldehyde
5-Hydroxyindole-acetate
5-Methoxyindole-acetate
5-Hydroxyindole-acetylglycine
5-Methoxytryptamine
Formyl-5-hydroxykynuramine
N-Methylserotonin
6-Hydroxyindolelactate
3-Methylindolepyruvate
Indole-3-ethanol
3-Methyldioxyindole
Indolelactate
Anthranilate
2-Formaminobenzoylacetate
Benzoate degradation via hydroxylation
Nicotinamide metabolism
Glucobrassicin
Cinnavalininate
Isophenoxazine
2-Aminophenol
2-Amino-3-carboxymuconate semialdehyde
Quinolinate
3-Hydroxyanthranilate
3-Methoxyanthranilate
8-Methoxykynurenate
Xanthurenate
2-Aminomuconate semialdehyde
2-Aminomuconate
7'-Oxalocrotonate
2-Oxoadipate
Glutaryl-CoA
Crotonyl-CoA
Acetoacetyl-CoA
(S)-3-Hydroxybutanoyl-CoA
4-(2-Amino-3-hydroxyphenyl)-2,4-dioxobutanoate
3-Hydroxykynurenine
3-Hydroxy-L-kynurenine
L-Kynurenine
N-Formylkynurenine
5-Hydroxy-N-formylkynurenine
5-Hydroxy-L-tryptophan
5-Hydroxyindolepyruvate
5-Hydroxyindole
Indoxyl
Acetylindoxyl
N-Acetylisatin
Isatin
2-Formylaminobenzaldehyde
3-Indoleglycolaldehyde
2,3-Dihydroxyindole
Tryptophan biosynthesis
Formylanthranilate
Anthranilate
Benzoate degradation via hydroxylation
2-Oxoglutarate
Oxaloacetate
5-(2'-Carboxyethyl)-4,6-dihydroxypicolinate
5-(2'-Formylethyl)-4,6-dihydroxypicolinate
5-(3'-Carboxy-3'-oxopropenyl)-4,6-dihydroxypicolinate
7,8-Dihydroxykynurenate
7,8-Dihydro-7,8-dihydroxykynurenate
4-(2-Aminophenyl)-2,4-dioxobutanoate
Kynurenate
4,8-Dihydroxyquinoline
5-Hydroxykynurenine
4-(2-Amino-5-hydroxyphenyl)-2,4-dioxobutanoate
6-Hydroxykynurenate
4,6-Dihydroxyquinoline
Glycolysis
Acetyl-CoA
00380 2/5/03

FIGURE 19

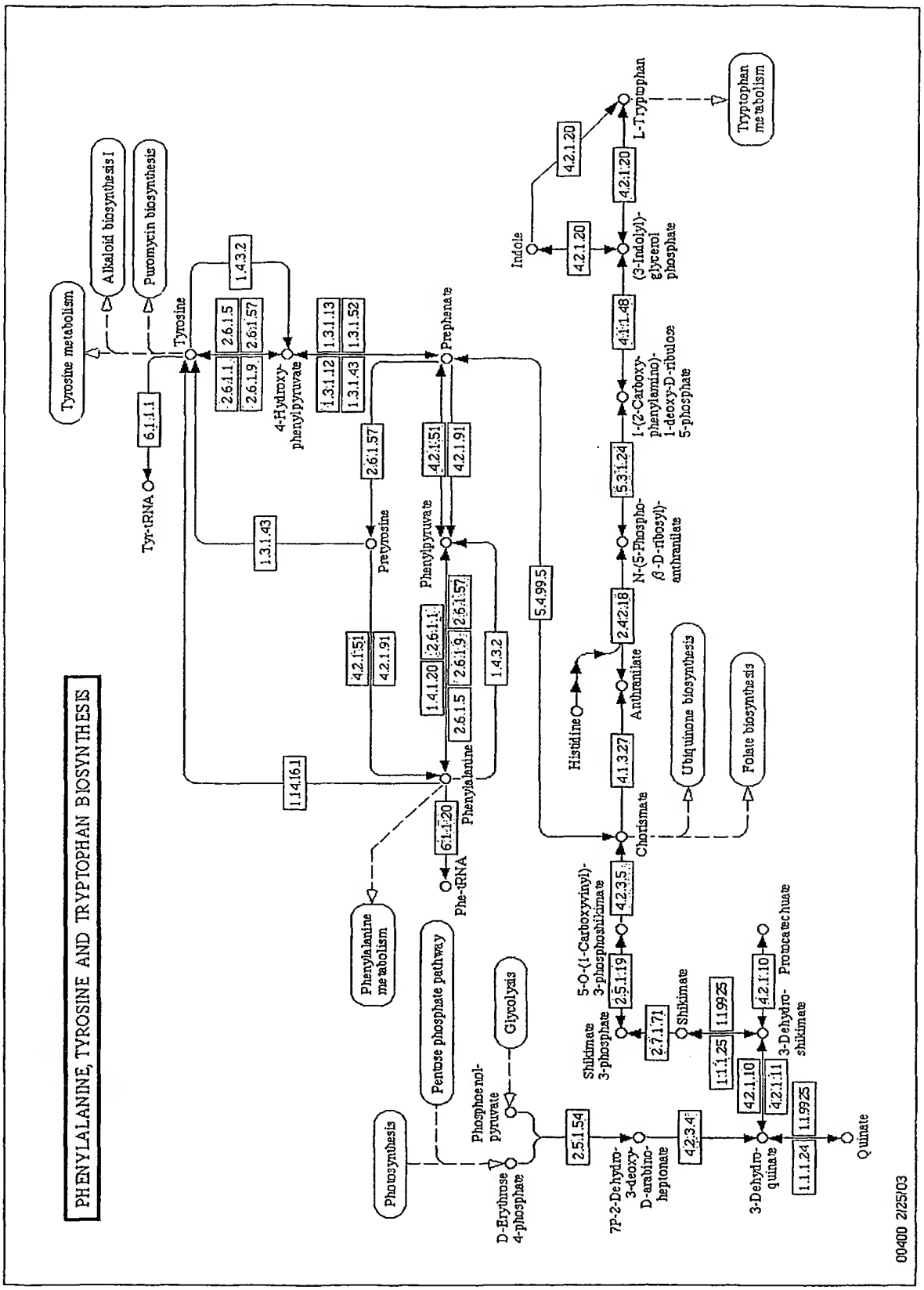

PHENYLALANINE, TYROSINE AND TRYPTOPHAN BIOSYNTHESIS
Tyrosine metabolism
Alkaloid biosynthesis I
Puromycin biosynthesis
Tyrosine
Tyr-tRNA
6.1.1.1
4-Hydroxy-phenylpyruvate
Prephenate
Prevnosine
Phenylpyruvate
Phenylalanine
Phe-tRNA
Phenylalanine metabolism
Pentose phosphate pathway
Glycolysis
Photosynthesis
Phosphoenol-pyruvate
D-Erythrose 4-phosphate
7P-2-Dehydro-3-deoxy-D-arabino-heptonate
3-Dehydro-quinate
Quinate
Shikimate
Shikimate 3-phosphate
5-O-(1-Carboxyvinyl)-3-phosphoshikimate
Chorismate
Anthranilate
Histidine
Ubiquinone biosynthesis
Folate biosynthesis
N-(5-Phospho-β-D-ribosyl)-anthranilate
1-(2-Carboxy-phenylamino)-1-deoxy-D-ribulose 5-phosphate
(3-Indolyl)-glycerol phosphate
Indole
L-Tryptophan
Tryptophan metabolism
3-Dehydro-shikimate
Protocatechuate
00400 2/25/03

FIGURE 20

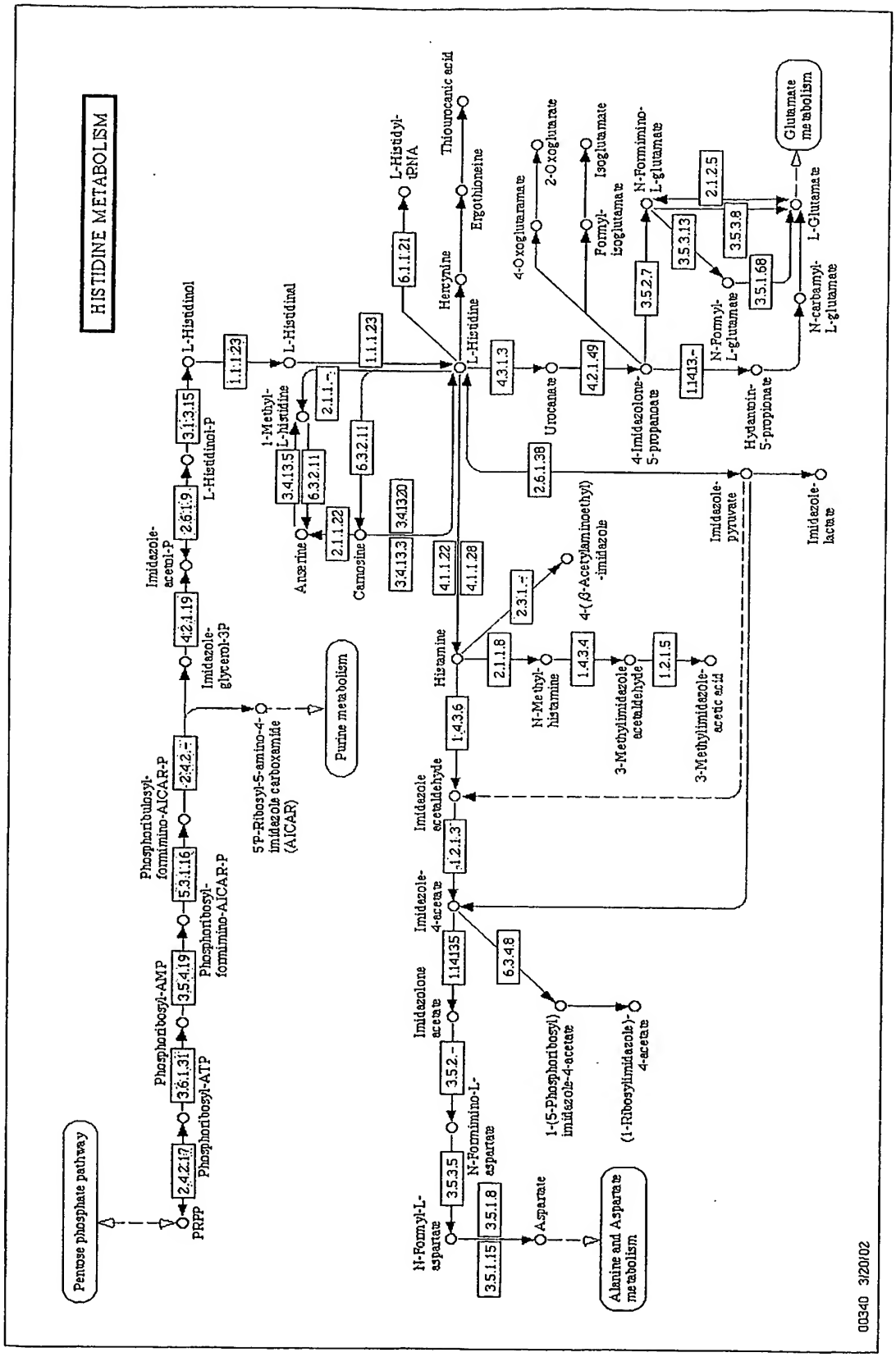

HISTIDINE METABOLISM
Pentose phosphate pathway
PRPP
2.4.2.17
Phosphoribosyl-ATP
3.6.1.31
Phosphoribosyl-AMP
3.5.4.19
Phosphoribosyl-formimino-AICAR-P
5.3.1.16
Phosphoribulosyl-formimino-AICAR-P
2.4.2.-
5'P-Ribosyl-5-amino-4-imidazole carboxamide (AICAR)
Purine metabolism
4.2.1.19
Imidazole-glycerol-3P
Imidazole-acetol-P
2.6.1.9
L-Histidinol-P
3.1.3.15
L-Histidinol
1.1.1.23
L-Histidinal
2.1.1.-
1-Methyl-L-histidine
3.4.13.5
6.3.2.11
Anserine
2.1.1.22
Carnosine
3.4.13.3
3.4.13.20
6.3.2.11
1.1.1.23
L-Histidyl-tRNA
6.1.1.21
Hercynine
L-Histidine
Thiourocanic acid
Ergothioneine
4.3.1.3
Urocanate
2.6.1.38
4-Imidazolone-5-propanoate
4.2.1.49
4-Oxoglutaramate
2-Oxoglutarate
Formyl-isoglutamate
Isoglutamate
3.5.2.7
N-Formimino-L-glutamate
1.14.13.-
N-Formyl-L-glutamate
3.5.3.13
3.5.1.68
2.1.2.5
3.5.3.8
N-carbamyl-L-glutamate
L-Glutamate
Glutamate metabolism
Hydantoin-5-propionate
Imidazole-pyruvate
Imidazole-lactate
4.1.1.22
4.1.1.28
Histamine
2.3.1.-
4-(β-Acetylaminoethyl)-imidazole
2.1.1.8
N-Methyl-histamine
1.4.3.4
3-Methylimidazole-acetaldehyde
1.2.1.5
3-Methylimidazole-acetic acid
1.4.3.6
Imidazole acetaldehyde
1.2.1.3
Imidazole-4-acetate
1.14.13.5
6.3.4.8
Imidazolone-acetate
3.5.2.-
N-Formimino-L-aspartate
3.5.3.5
3.5.1.8
N-Formyl-L-aspartate
3.5.1.15
Aspartate
Alanine and Aspartate metabolism
1-(5-Phosphoribosyl)-imidazole-4-acetate
(1-Ribosylimidazole)-4-acetate
00340 3/20/02

FIGURE 21

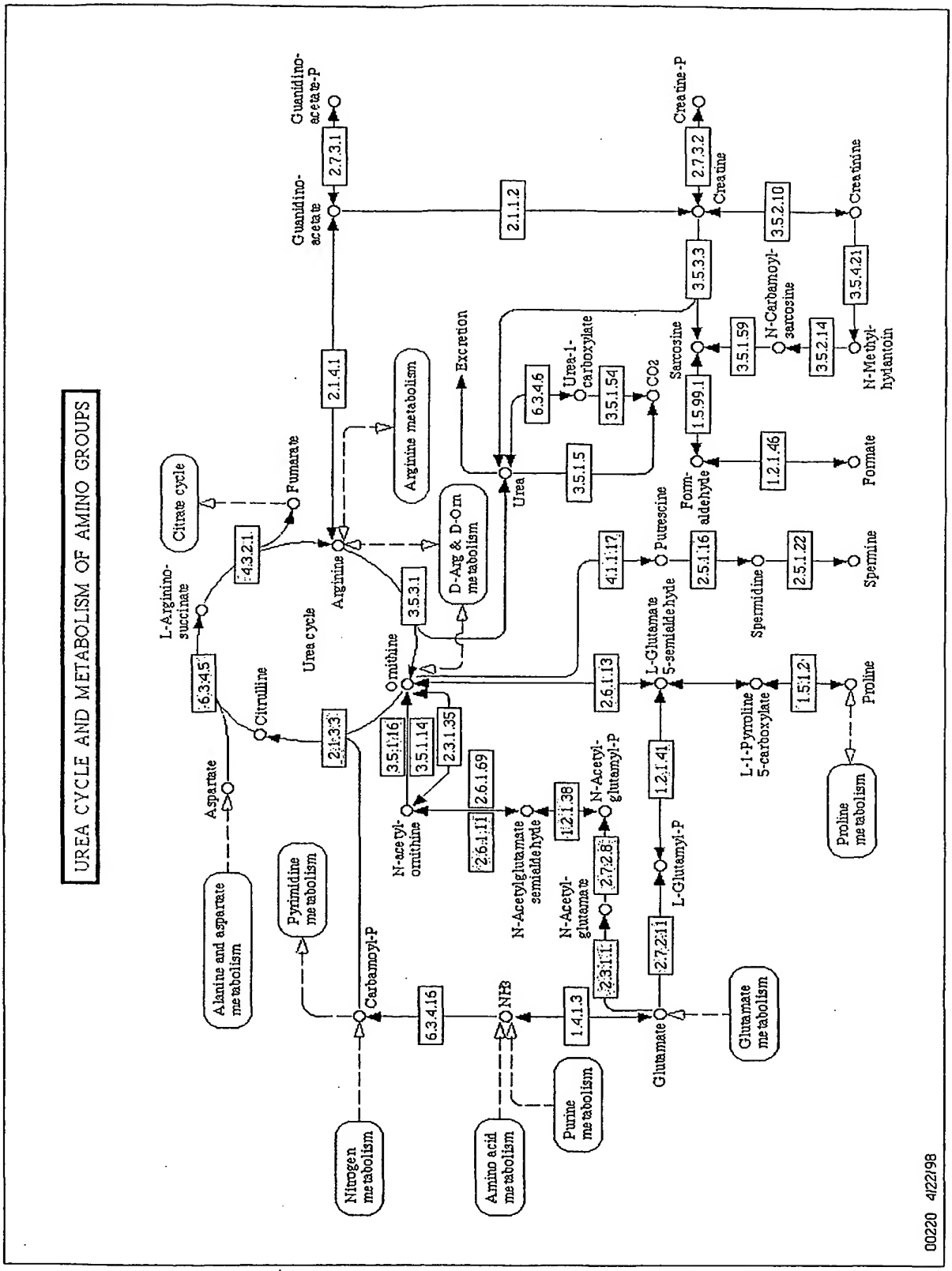

UREA CYCLE AND METABOLISM OF AMINO GROUPS
Guanidino-acetate-P
2.7.3.1
Guanidino-acetate
2.1.1.2
Creatine-P
2.7.3.2
Creatine
3.5.2.10
Creatinine
3.5.3.3
Sarcosine
3.5.1.59
N-Carbamoyl-sarcosine
3.5.2.14
3.5.4.21
N-Methyl-hydantoin
Excretion
6.3.4.6
Urea-1-carboxylate
3.5.1.54
CO2
1.5.99.1
Form-aldehyde
1.2.1.46
Formate
Urea
3.5.1.5
D-Arg & D-Orn metabolism
Arginine metabolism
2.1.4.1
Fumarate
Citrate cycle
4.3.2.1
L-Arginino-succinate
Arginine
3.5.3.1
Urea cycle
6.3.4.5
Citrulline
Ornithine
2.1.3.3
4.1.1.17
Putrescine
2.5.1.16
Spermidine
2.5.1.22
Spermine
2.6.1.13
L-Glutamate 5-semialdehyde
1.5.1.12
L-1-Pyrroline 5-carboxylate
Proline
Proline metabolism
Aspartate
Alanine and asparate metabolism
3.5.1.16
3.5.1.14
2.3.1.35
2.6.1.69
2.6.1.11
N-acetyl-ornithine
N-Acetylglutamate semialdehyde
1.2.1.38
N-Acetyl-glutamate-P
1.2.1.41
2.7.2.8
N-Acetyl-glutamate
2.3.1.1
L-Glutamyl-P
2.7.2.11
Pyrimidine metabolism
Carbamoyl-P
6.3.4.16
NH3
1.4.1.3
Glutamate
Glutamate metabolism
Nitrogen metabolism
Amino acid metabolism
Purine metabolism
00220 4/22/98

FIGURE 22

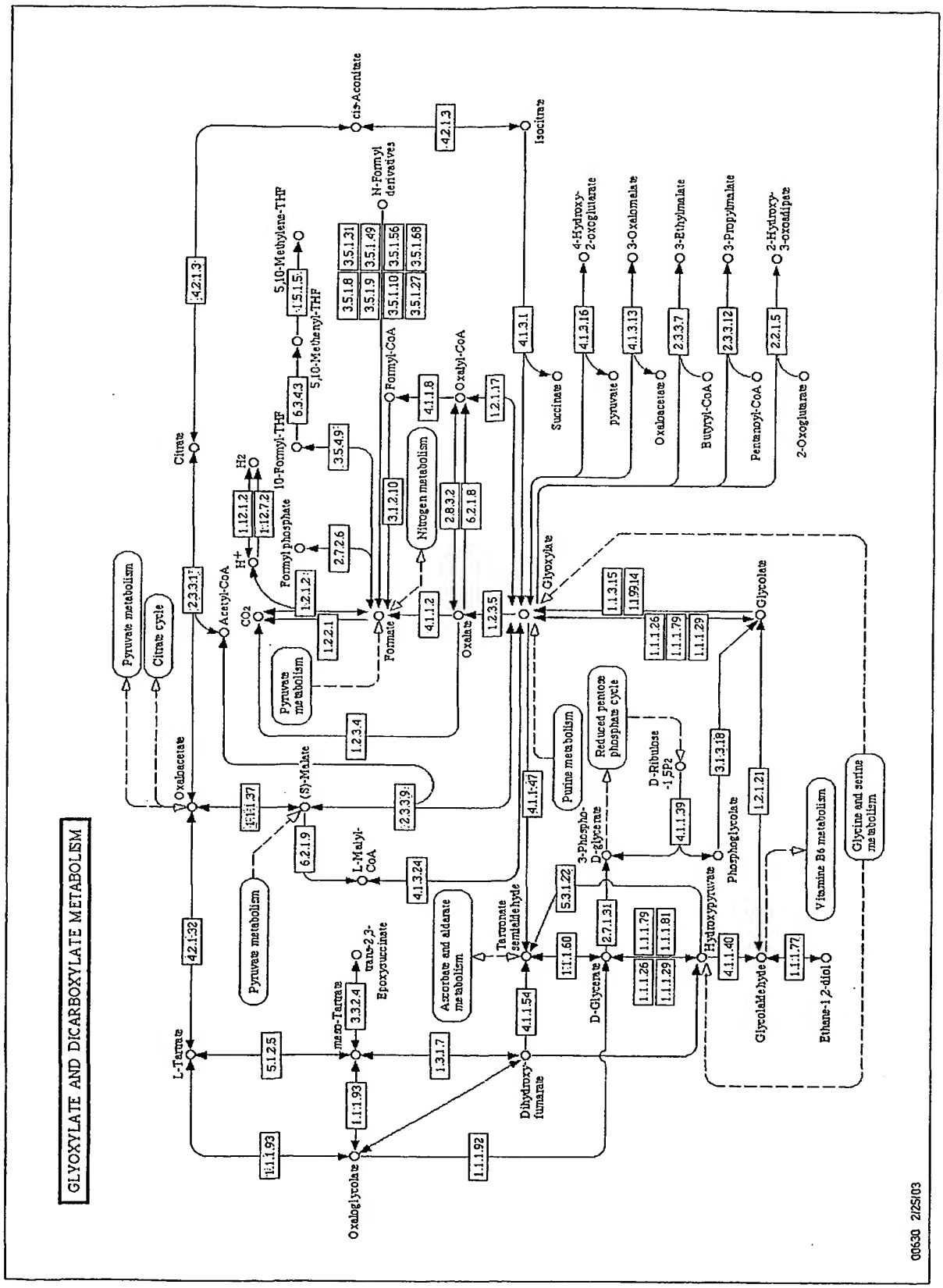
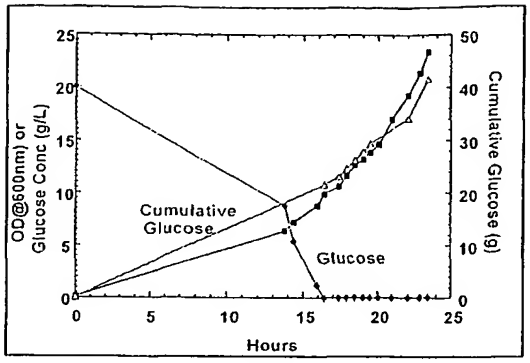
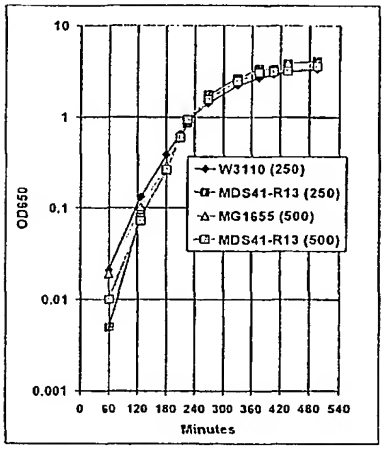
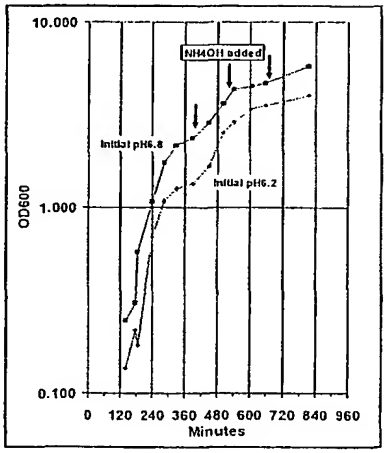

GLYOXYLATE AND DICARBOXYLATE METABOLISM
00630 2/25/03
Pyruvate metabolism
Citrate cycle
Oxaloacetate
Acetyl-CoA
Citrate
cis-Aconitate
Isocitrate
Succinate
Fumarate
(S)-Malate
L-Malyl-CoA
Glyoxylate
Formate
Oxalate
Formyl-CoA
Oxalyl-CoA
Formyl phosphate
10-Formyl-THF
5,10-Methenyl-THF
5,10-Methylene-THF
N-Formyl derivatives
Nitrogen metabolism
Pyruvate metabolism
Reduced pentose phosphate cycle
Purine metabolism
D-Ribulose -1,5P2
3-Phospho-D-glycerate
D-Glycerate
Tartronate semialdehyde
Hydroxypyruvate
Phosphoglycolate
Glycolate
Glycolaldehyde
Ethane-1,2-diol
Vitamine B6 metabolism
Glycine and serine metabolism
Ascorbate and aldarate metabolism
Dihydroxy-fumarate
L-Tartrate
meso-Tartrate
trans-2,3-Epoxysuccinate
Oxalglycolate
4-Hydroxy-2-oxoglutarate
3-Oxalomalate
3-Ethylmalate
3-Propylmalate
2-Hydroxy-3-oxoadipate
pyruvate
Oxaloacetate
Butyryl-CoA
Pentanoyl-CoA
2-Oxoglutarate
2.3.3.1
4.2.1.3
4.1.3.16
4.1.3.13
2.3.3.7
2.3.3.12
2.2.1.5
4.1.3.1
1.2.1.17
4.1.1.8
3.5.4.9
6.3.4.3
1.5.1.5
3.5.1.8
3.5.1.9
3.5.1.31
3.5.1.49
3.5.1.10
3.5.1.56
3.5.1.27
3.5.1.68
1.12.1.2
1.12.7.2
2.7.2.6
3.1.2.10
2.8.3.2
6.2.1.8
1.2.1.2
1.2.2.1
4.1.1.2
1.2.3.5
1.2.3.4
1.1.3.15
1.1.9.14
1.1.1.26
1.1.1.79
1.1.1.29
4.1.1.47
3.1.3.18
1.2.1.21
4.1.1.39
5.3.1.22
2.7.1.31
1.1.1.60
1.1.1.79
1.1.1.81
1.1.1.26
1.1.1.29
4.1.1.40
1.1.1.77
4.1.1.54
1.3.1.7
5.1.2.5
4.2.1.32
3.3.2.4
1.1.1.93
1.1.1.92
6.2.1.9
4.1.3.24
2.3.3.9
1.1.1.37

FIGURE 23


OD@600nm) or Glucose Conc (g/L)
Cumulative Glucose (g)
Cumulative Glucose
Glucose
Hours


OD650
W3110 (250)
MDS41-R13 (250)
MG1655 (500)
MDS41-R13 (500)
Minutes


OD600
NH4OH added
Initial pH6.8
Initial pH6.2
Minutes

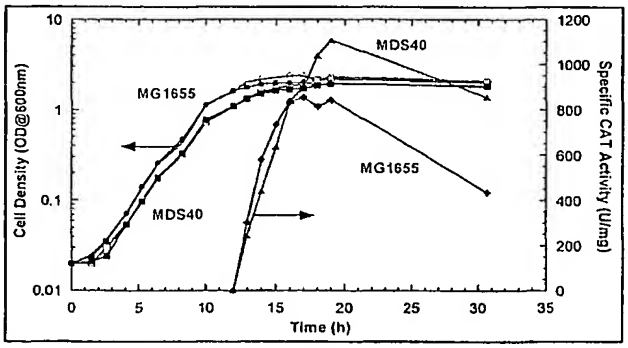

Cell Density (OD@600nm)
Specific CAT Activity (U/mg)
MDS40
MG1655
MG1655
MDS40
Time (h)

FIGURE 24

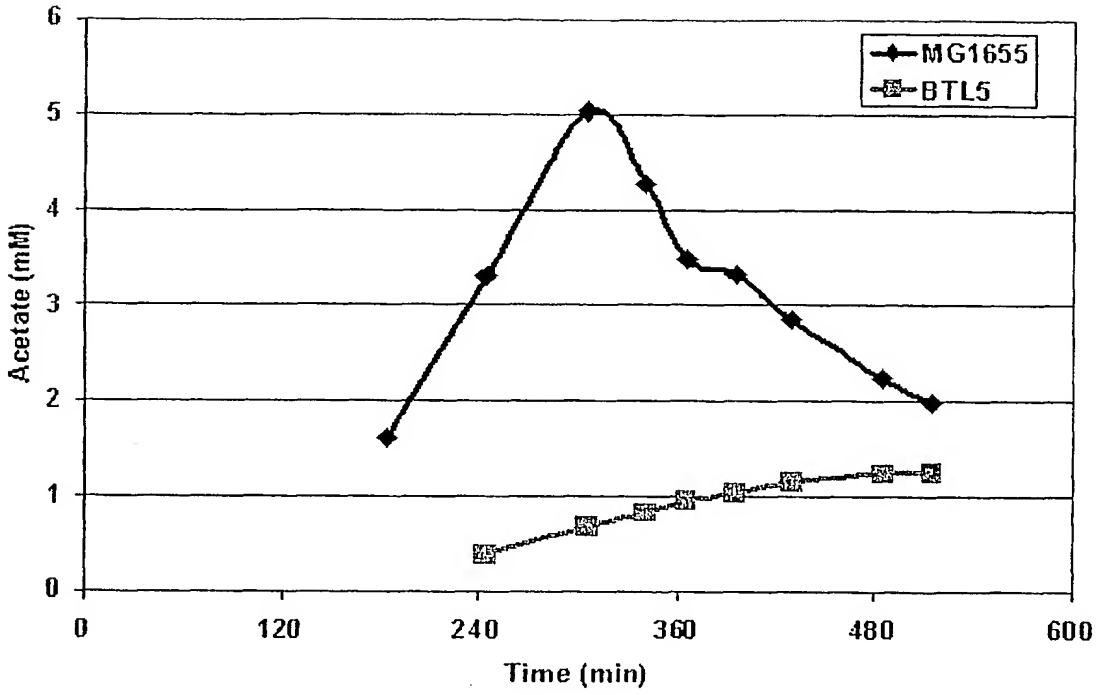
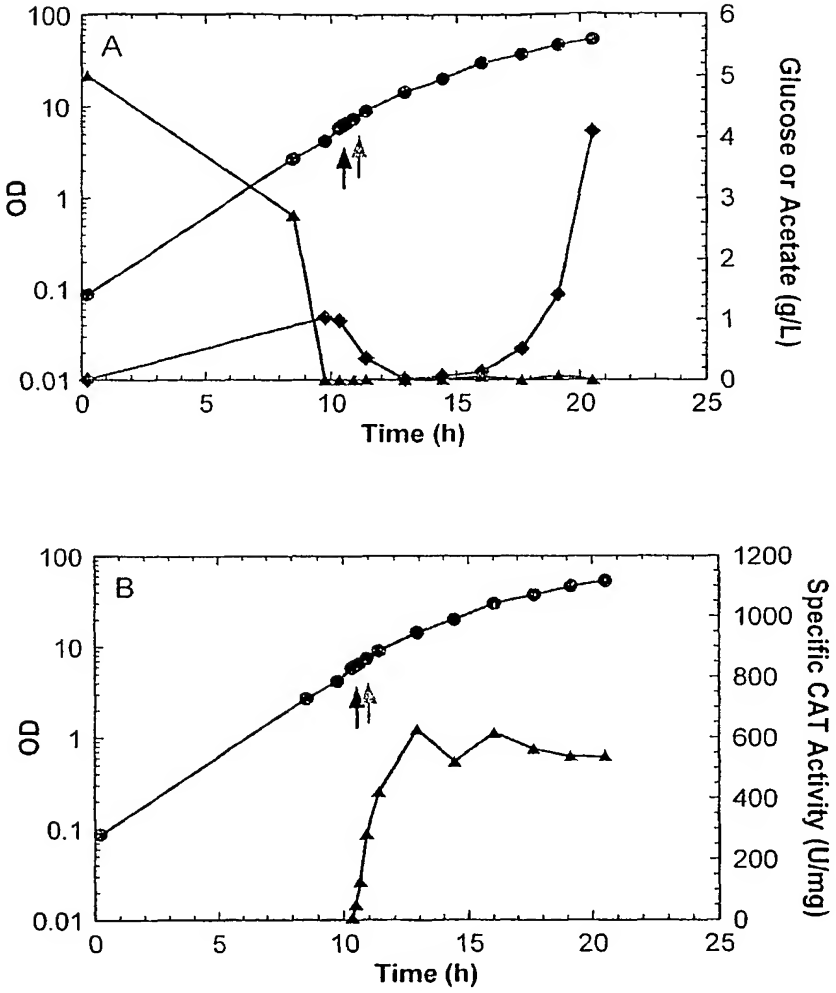
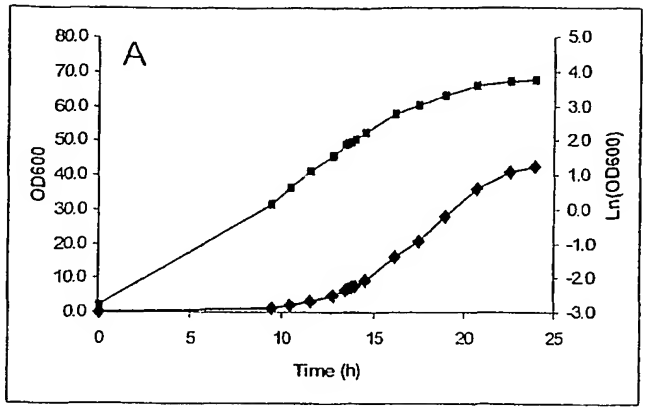


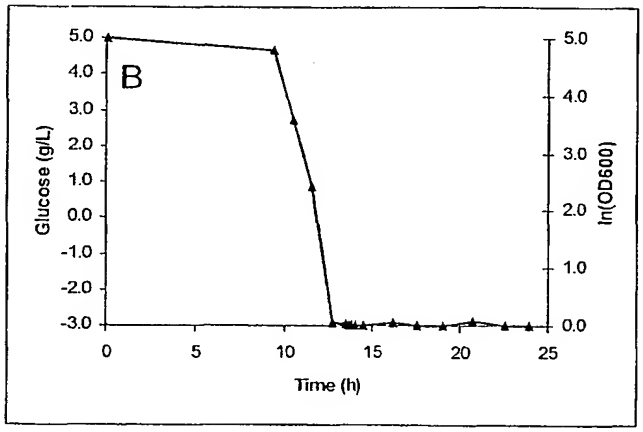
FIGURE 25

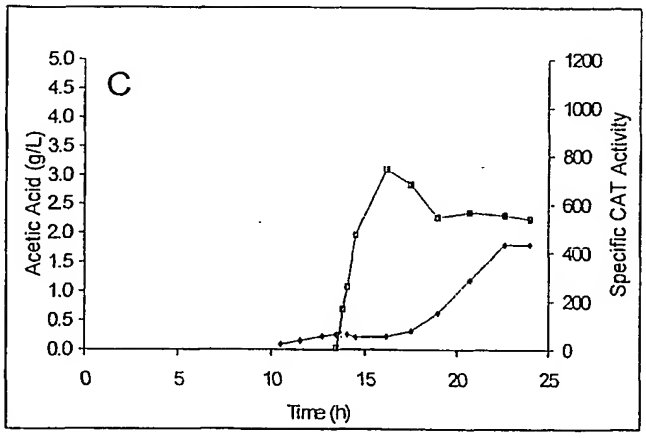


MDS42

FIGURE 26

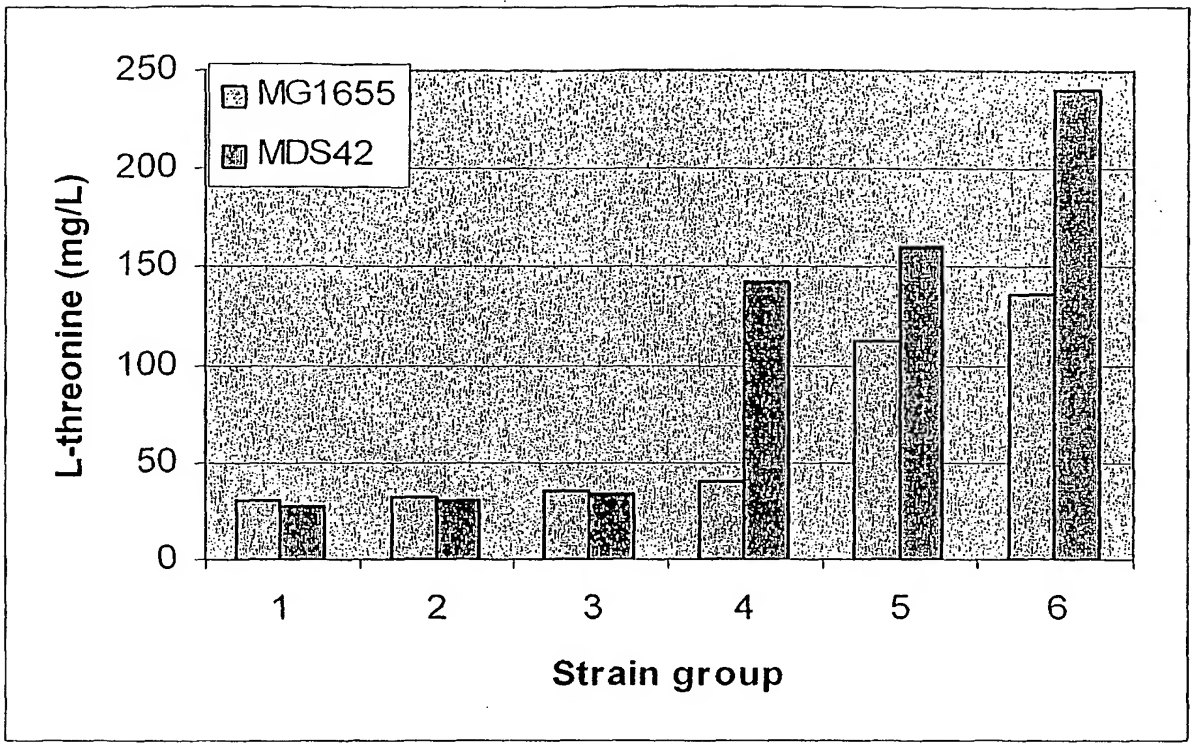






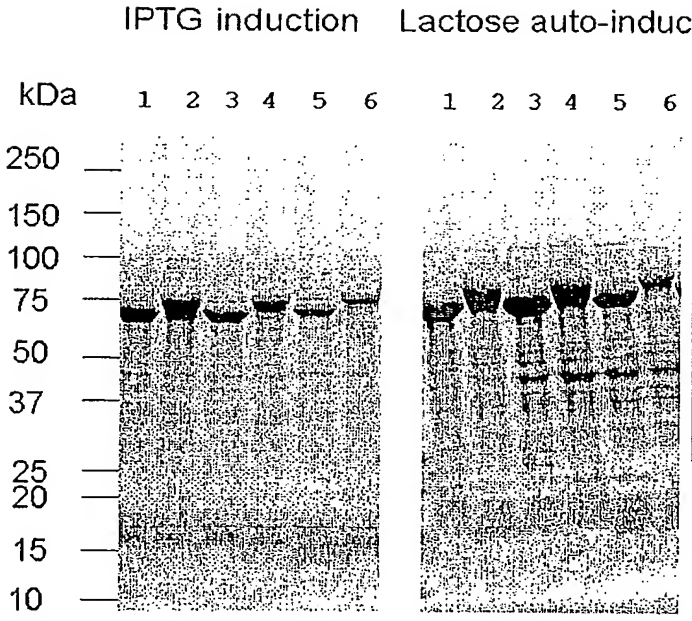
MDS42-pta

FIGURE 27



1: Wild-type MG1655 and MDS42
2: *Ptac-thrA*BC*
3: *Ptac-thrA*BC, lacI::tetA*
4: *Ptac-thrA*BC, lacI::tetA, tdh::CAT*
5: *Ptac-thrA*BC, lacI::tetA, tdh::CAT, rhtA23(1 copy)*
6: *Ptac-thrA*BC, lacI::tetA, tdh::CAT, rhtA23[pTOPOCR] (16 copies)*

FIGURE 28



Lane	Clone	Strain
1	A	MDS42
2	B	MDS42
3	A	MG1655Δtdh
4	B	MG1655Δtdh
5	A	MDS42Δtdh
6	B	MDS42Δtdh

FIGURE 29

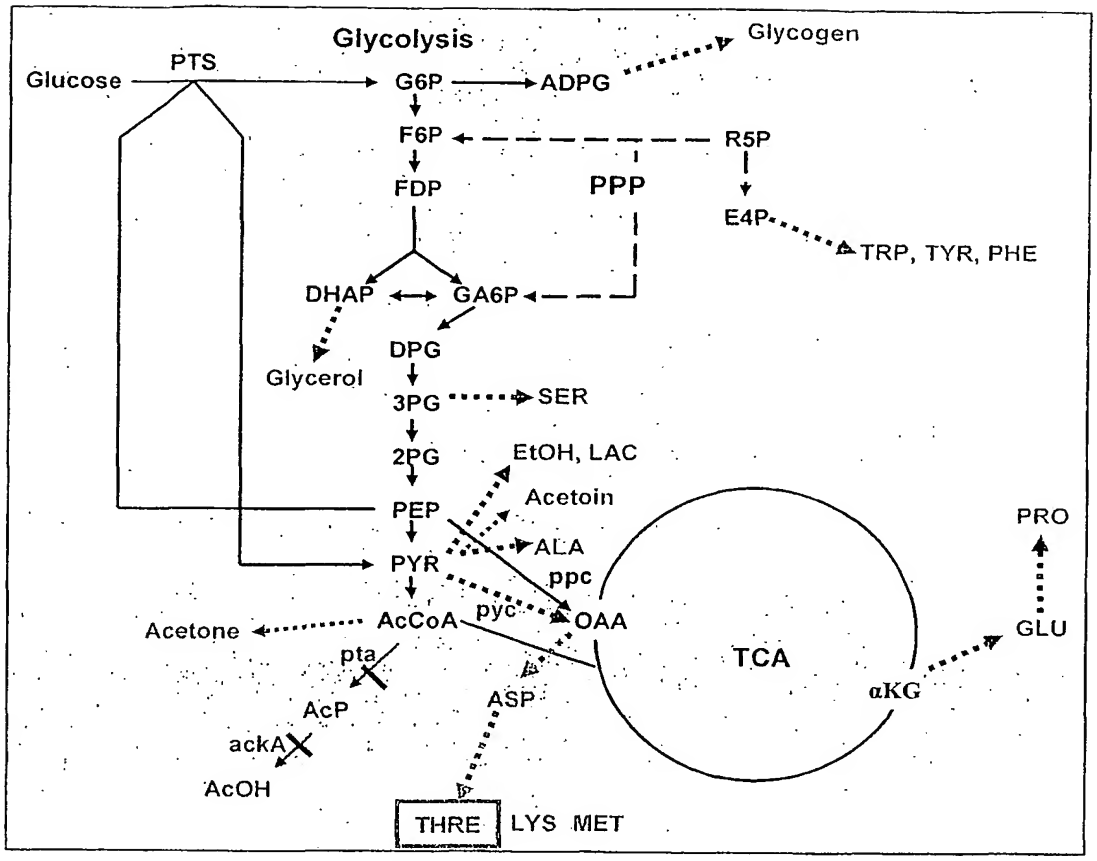
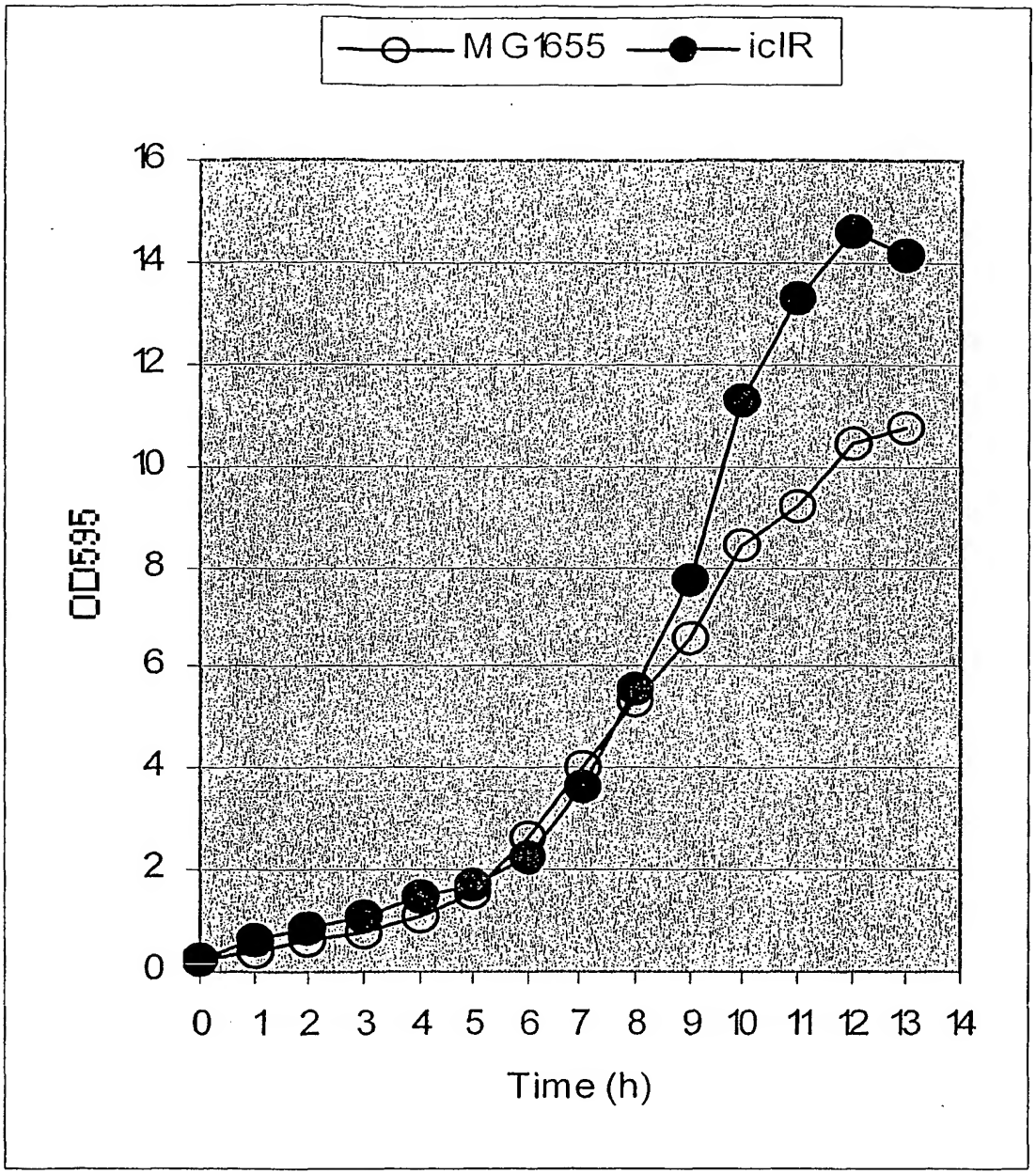

Glycolysis
Glycogen
PTS
Glucose
G6P
ADPG
F6P
R5P
FDP
PPP
E4P
TRP, TYR, PHE
DHAP
GA6P
Glycerol
DPG
3PG
SER
2PG
EtOH, LAC
Acetoin
PEP
ALA
PRO
PYR
ppc
pyc
OAA
Acetone
AcCoA
TCA
GLU
pta
αKG
AcP
ASP
ackA
AcOH
THRE
LYS
MET

FIGURE 30


MG1655
iclR
OD595
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (h)